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**14. ABSTRACT**

Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants which have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, the following specific aims are proposed: 1. To generate and characterize models of early life stress. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from models developed in Specific Aim 1 are exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatment with the SSRI sertraline.

**15. SUBJECT TERMS**
Rats, prenatal stress, PTSD, open field test, social interaction test, fear conditioning, extinction, glucocorticoid receptors, norepinehrine.

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## A. INTRODUCTION:

Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants that have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, we proposed the following specific aims: 1. To generate and characterize animal models of early life stress. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from animal models developed in Specific Aim 1 are exposed to a traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatments with the SSRI sertraline in trauma-exposed adults.

## B. BODY:

Following from results reported in the progress report last year, we have completed a series of experiments designed to determine if prenatal CORT exposure was sufficient to recreate the previously described effects of PNS on fear extinction learning and stress neurobiology that we reported previously (Green, Rani et al. 2011). Specifically, we finalized results from Model 3, Tasks 1 and 2, Steps 4 and 5 in which we exposed rats to corticosterone (CORT) in utero to mimic the levels of CORT observed in prenatal stress. The behavioral assays were substantially completed at the end of last year and we produced preliminary results on neurochemical and molecular biological assays on the brain tissues from those rats. During the past year we completed the neurochemical/molecular biological assays by adding more samples. The completed work was published on August 6, 2013 (Bingham, Sheela Rani et al. 2013). Please see the attached reprints. Note that testing of Model 4, i.e. treatment of prenatally stressed dams with the CORT synthesis blocker, Metyrapone, was discontinued because of adverse effects on maternal and fetal survival.

To summarize the behavioral experiments, pregnant female rats were delivered and divided into 3 prenatal treatment groups (vehicle controls, PNS, or CORT) and a subset of their male offspring were subject to control or CAPS stress from p46-p60. These animals (n=15/group) were then tested in the fear conditioning and extinction paradigm following the end of CAPS stress. We found that both prenatal CORT and adult CAPS stress independently delayed extinction learning while prenatal CORT treatment impairs the retention of extinction learning. We worked with Jim Mintz, PhD (Director of the Data Analysis Core) to refine our novel analysis of the extinction behavior using single exponential decay fit. We developed a statistical

analysis that better allows us to compare the different phases of fear extinction behavior between groups. Analysis of the decay constants derived from the regression lines indicate that both prenatal CORT and adult CAPS stress reduced the rate of extinction learning. By contrast, analysis of the plateau value indicates no effect of either prenatal CORT or CAPS on the final level of freezing behavior displayed at the end of the extinction learning session. With respect to the regression analysis of extinction retention curves, Analysis of the decay constants indicates that all groups showed equivalent rates of extinction re-learning. However, analysis of the plateau term indicated that rats exposed to prenatal CORT treatment were unable to re-extinguish to the same final level of freezing behavior as controls. In sum, these data demonstrate that prenatal CORT exposure, similar to prenatal stress, impairs the extinction of learned fear in an additive and distinct manner from adult exposure to CAPS stress.

With respect to the neurobiological consequences of PNS or prenatal CORT, sibling male offspring from the animals described above were allowed to grow to adulthood undisturbed, at which point they were sacrificed and the medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and a section of the rostral pons containing the locus coeruleus (LC) were dissected. Both PNS and prenatal CORT treatment decreased glucocorticoid receptor protein levels in the mPFC, hippocampus, and hypothalamus when compared to control offspring. Both treatments also decreased tyrosine hydroxylase levels in the LC. Prenatal CORT also resulted in a small, but significant decrease in hippocampal BDNF expression. These behavioral and neurobiological results were published online August 6, 2013 in *Psychoneuroendocrinology* (Bingham, Sheela Rani et al. 2013).

During the past year, we also began work on Specific Aim 4, i.e. Task 3, to determine the efficacy of SSRI treatment in the PTSD model that we developed. These studies are not completed, but we applied for and we were approved for a no-cost extension to complete the work. As we planned these experiments, we realized that because of the inherent variability in extinction behavior, the addition of 2 treatment groups to our experimental design would require an uncomfortably large sample size per group to render sufficient statistically power to discriminate treatment effects. Therefore, we designed an experiment to increase the effect size of CAPS stress through repeated administration. In this experiment, the animals were fear conditioned previous to the stressor to allow us to directly investigate the effects of stress on extinction processes without the conflict of stress effects on fear acquisition. We then administered the 15-day CAPS stress twice, with a single tone-shock “reminder” given on the day between the 2 CAPS sessions. To allow a potential treatment window, testing in fear extinction occurred 5 days after the end of stress. As this was a pilot experiment to examine effect size, the sample size per group was smaller than our previously published experiments. Even so, we found that CAPS stress produced a trend to an effect of stress ($p=0.16$) on extinction learning with an effect size similar to those we have demonstrated previously (Figure 1: approx. 20% freezing vs 40% freezing on tones 4-7). Interestingly, we found that this repeated stress procedure produced a trend to an effect of stress ($p=0.07$) and a stress x tone interaction ($p=0.09$) on the retention of extinction learning. The impairment in retention of extinction learning produced by repeated CAPS was a new effect of CAPS that we think more closely models the deficits in fear extinction that occur in PTSD patients.

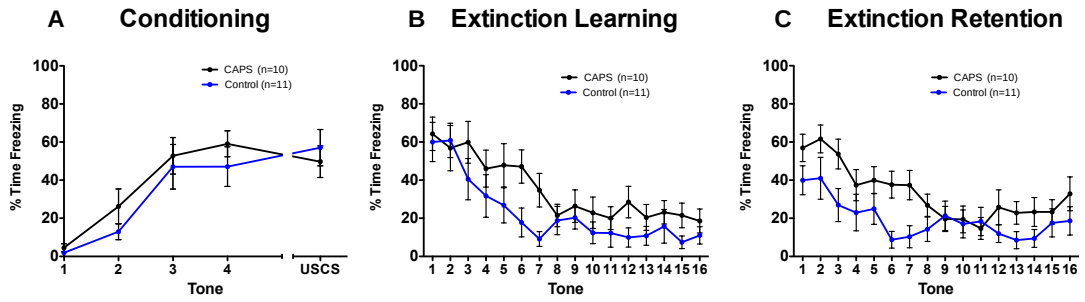


**Figure 1 . Effect of repeated CAPS stress on fear extinction learning and retention. A) All animals were conditioned previous to any manipulation and were divided into groups so that there were no differences in acquisition of freezing behavior. In addition, there were no significant differences in freezing during the "reminder" tone-shock pairing administered between sessions of CAPS stress. B) Repeated CAPS stress produced a trend to increased freezing during extinction learning. C) Repeated CAPS stress produced a trend to increased freezing during the extinction test as well as a trend to a tone by stress interaction.**

While the effect on extinction is a new and exciting finding, we are still investigating other factors that may allow us to increase the effect sizes. We will test the possibility that by decreasing the time between the end of stress and extinction testing from 5 days to 3 days, in combination with repeated CAPS stress, we will be able to produce a stress model that we can use to investigate antidepressant treatments. These experiments are currently ongoing and will be finished in the next year.

## REFERENCES

Bingham, B., C. Sheela Rani, et al. "Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior." Psychoneuroendocrinology, August 9, 2013 [Epub ahead of print].

Green, M. K., C. S. Rani, et al. "Prenatal stress induces long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress." Neuroscience, 2011 Sep 29;192:438-51.

## KEY RESEARCH ACCOMPLISHMENTS:

- **During the reporting period, we have completed the neurochemical studies of prenatal corticosterone (Model 3) and the data were published online on August 6, 2013 in the journal Neuroendocrinology.**
- **That paper reports that prenatal corticosterone treatment produces a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex. We also found that, like prenatal stress, prenatal corticosterone reduced TH mRNA, suggesting that prenatal stress produces its effects on TH mRNA through a mechanism dependent on corticosterone.**

- **We have begun preliminary studies to examine the effects of SSRIs. We have begun by modifying our procedures to increase the effect size and reduce the number of animals required for testing.**

**REPORTABLE OUTCOMES:**

During the reporting period, work by our research team was published by the journal *Neuropharmacology* in the November 2012 edition. A copy is attached in the appendix of this report.

- Roth, M. K., Bingham, B., Shah, A., Joshi, A., Frazer, A., Strong, R., & Morilak, D. A.; for the STRONG STAR Consortium. (2012). Effects of Chronic Plus Acute Prolonged Stress on measures of coping style, anxiety, and evoked HPA-axis reactivity. *Neuropharmacology*, *63*(6), 1118-1126.

During the reporting period, work by our research team was also published online August 6, 2013 in the journal Psychoneuroendocrinology. A copy is attached in the appendix of this report.

- Bingham BC, Sheela Rani CS, Frazer A, Strong R, Morilak DA. Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior. *Psychoneuroendocrin.* 2013 Aug 9. [Epub ahead of print]

**CONCLUSION:**

During the reporting period, we finished testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring. We confirmed that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex and hippocampus and reduced TH mRNA in the locus ceruleus. This work has led to the publication of a substantive paper on the effects of prenatal corticosterone on susceptibility to stress in adult offspring (Bingham et al., 2013). During the remainder of the past year we continued to develop the CAPS model of PTSD so that we can begin testing SSRIs. We found that modifications to the procedures have increased the effect size and thus reduced the number of animals required for these studies. We plan to complete the studies in the next year on the no-cost extension.

**APPENDICES:**

**Reprint:** Roth, M. K., Bingham, B., Shah, A., Joshi, A., Frazer, A., Strong, R., & Morilak, D. A.; for the STRONG STAR Consortium. Effects of Chronic Plus Acute Prolonged Stress on measures of coping style, anxiety, and evoked HPA-axis reactivity. *Neuropharmacology*, November. 2012 *63*(6), 1118-1126.

**Reprint:** Bingham BC, Sheela Rani CS, Frazer A, Strong R, Morilak DA.Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior. Psychoneuroendocrin. 2013 Aug 9. [Epub ahead of print]

**SUPPORTING DATA:** Shown in the body of the report (pp. 4-6) and in the attached reprints.

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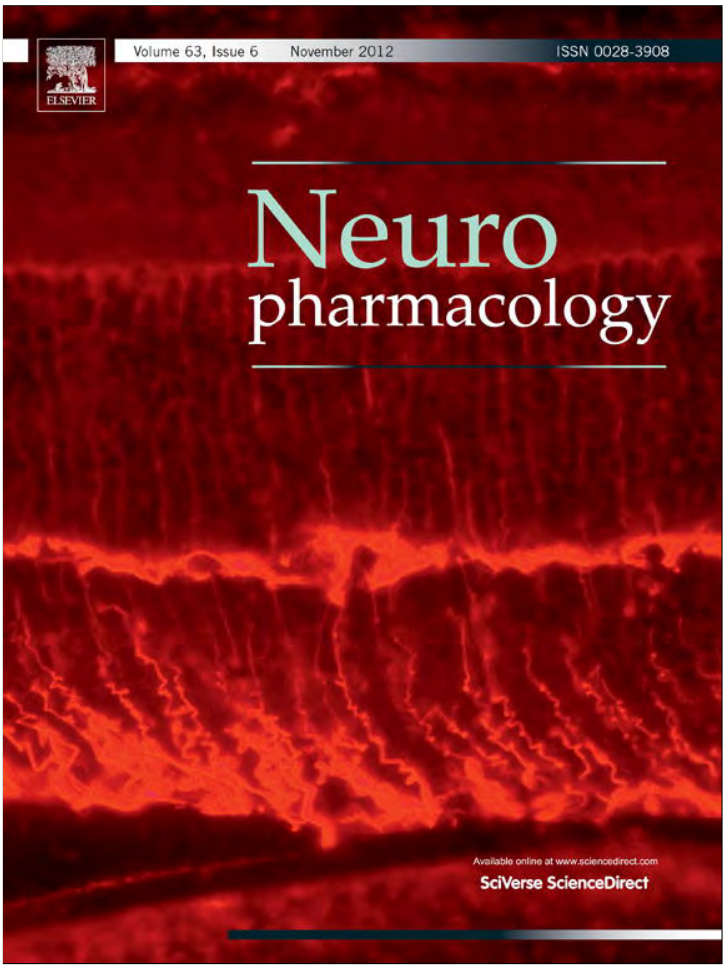


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# Effects of chronic plus acute prolonged stress on measures of coping style, anxiety, and evoked HPA-axis reactivity

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ABSTRACT

Exposure to psychological trauma is the precipitating factor for PTSD. In addition, a history of chronic or traumatic stress exposure is a predisposing risk factor. We have developed a Chronic plus Acute Prolonged Stress (CAPS) treatment for rats that models some of the characteristics of stressful events that can lead to PTSD in humans. We have previously shown that CAPS enhances acute fear responses and impairs extinction of conditioned fear. Further, CAPS reduced the expression of glucocorticoid receptors in the medial prefrontal cortex. In this study we examined the effects of CAPS exposure on behavioral stress coping style, anxiety-like behaviors, and acute stress reactivity of the hypothalamic–pituitary–adrenal (HPA) axis. Male Sprague-Dawley rats were exposed to CAPS treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/day, 14 days) followed on day 15 by a single 1-h session of sequential acute stressors (social defeat, immobilization, swim). After CAPS or control treatment, different groups were tested for shock probe defensive burying, novelty suppressed feeding, or evoked activation of adrenocorticotropic hormone (ACTH) and corticosterone release by an acute immobilization stress. CAPS resulted in a decrease in active burying behavior and an increase in immobility in the shock probe test. Further, CAPS-treated rats displayed increases in the latency to feed in the novelty suppressed feeding test, despite an increase in food intake in the home cage. CAPS treatment also reduced the HPA response to a subsequent acute immobilization stress. These results further validate CAPS treatment as a rat model of relevance to PTSD, and together with results reported previously, suggest that CAPS impairs fear extinction, shifts coping behavior from an active to a more passive strategy, increases anxiety, and alters HPA reactivity, resembling many aspects of human PTSD.

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## 1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling illness that occurs after exposure to a severe stress, e.g., a life-threatening event or witnessing such an event. PTSD is characterized by three classes of symptoms: re-experiencing, avoidance, and hyper-arousal (American Psychiatric Association, 2000). Re-experiencing involves intrusions of vivid memories and dreams, and even dissociations, related to the traumatic event. Avoidance of situations or stimuli that serve as reminders of the traumatic event may also be manifest as a general emotional and social detachment. Hyper-arousal is expressed as elevated anxiety, enhanced startle, irritability, sleep disturbance, and difficulty concentrating. Rape, physical attacks or abuse, threats with a weapon, and combat are some of the events typically associated with PTSD (Kessler et al., 1995). Chronic PTSD represents a significant health concern, not only because of the disabling nature of the symptoms, but also because of the long-term consequences on physical health, including higher rates of chronic disease, such as cardiovascular disease, diabetes, asthma, and obesity, as well as higher rates of

*Abbreviations:* ACTH, adrenocorticotropic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; mPFC, medial prefrontal cortex; NSFT, novelty suppressed feeding test; PD, postnatal day; PTSD, post-traumatic stress disorder; SPS, single prolonged stress.

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substance abuse (Centers for Disease Control and Prevention, 2006; Sareen et al., 2005). Chronic stress is also a risk factor, and possibly a causal factor, in the development of depressive and anxiety disorders (Breslau et al., 1999; Gilmer et al., 2005; Jordanova et al., 2007; Kendler et al., 1999; Koenen et al., 2007, 2002).

The complex nature of stress is particularly salient in wartime situations, in which there is a chronic state of environmental stress punctuated by intense, acute traumatic events. To model this, we developed a stress treatment that we have termed Chronic plus Acute Prolonged Stress (CAPS; not to be confused with the "CAPS" assessment used in human PTSD research). CAPS treatment combines 14-days of exposure to a chronic mild environmental stressor (chronic intermittent cold stress), followed on day 15 by a single session of intense acute stressors adapted from the Single Prolonged Stress (SPS) model (Yamamoto et al., 2009).

We have shown previously that CAPS treatment impaired fear extinction (Green et al., 2011), arguably an important component of human PTSD that may contribute to treatment resistance. For instance, PTSD patients show impairments in extinction (Blechert et al., 2007; Wessa and Flor, 2007), and they are incapable of suppressing fear responses in the presence of a safety signal, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). We also showed that CAPS treatment resulted in a down-regulation of glucocorticoid receptors (GR) in the medial prefrontal cortex (mPFC) (Green et al., 2011). This could have contributed to the impairments observed during extinction testing, as glucocorticoids are known to be involved in learning and memory, including fear and extinction learning (Gourley et al., 2009; de Quervain et al., 2009; Roozendaal et al., 2004, 2006). The mPFC, particularly the infralimbic cortex, is a key region involved in extinction learning (Milad and Quirk, 2002; Milad et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Sutker et al., 1995). Furthermore, humans with PTSD display dysregulated hypothalamic–pituitary–adrenal (HPA) axis activity, although the nature of this dysregulation remains unclear (Boscarino, 1996; Hoffman et al., 1989; Lemieux and Coe, 1995; Mason et al., 1986; Pitman and Orr, 1990; Yehuda et al., 1995, 1993, 1990).

Having defined some key components of PTSD in this model, in the present experiments, we continued to explore the effects of CAPS on other measures of PTSD-like symptomotology, including coping style/defensive behavior and generalized anxiety, as well as acute HPA stress reactivity. In our previous study, we observed that CAPS, particularly when combined with early life stress, resulted in persistent freezing during fear conditioning, at a point when other rats were shifting to a more active escape strategy (rearing and jumping) (Green et al., 2011). Thus, using the shock probe-defensive burying test in the present experiment, we tested the hypothesis that CAPS would produce a shift from an active coping strategy (burying) to a passive coping strategy (immobility). Likewise, we examined if CAPS would increase anxiety-like behavior in the novelty suppressed feeding test (NSFT). Finally, we tested if CAPS treatment produced changes in the HPA response evoked by an acute stressor.

## 2. Experimental procedures

### 2.1. Animals

In total, 103 adult male Sprague-Dawley rats were used in these experiments. The rats were born in our animal facility, and after weaning, they were pair-housed with a same-sex littermate until postnatal day (PD) 46–60, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages (25 × 45 × 15 cm) on a 12/12 h light–dark cycle (lights on at 07:00) with food and water available *ad libitum*. In addition, for the social defeat procedure, 12 adult male Long-Evans rats (Harlan, Indianapolis, IN), weighing at least 400 g, were used as defeaters. They were housed, together with an ovariectomized female, in large resident cages (60 × 60 × 35 cm) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

### 2.2. CAPS

CAPS treatment consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their home cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min swim stress. For social defeat, the ovariectomized Long-Evans female was removed from the resident cage, and the test rat was placed in the cage with the resident Long-Evans male rat. Typically within 10–30 s, the resident would attack and defeat the smaller "intruder" Sprague-Dawley test rat. Once defeat occurred, defined by the test rat assuming a supine posture and the resident showing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting it from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement, for 30 min. Finally, swim stress was accomplished by placing the rat in a cylindrical tank (30 cm diameter × 60 cm height) filled to a depth of 30 cm with water at approximately 23 °C. Control rats were handled briefly for approximately 30 s.

### 2.3. Experiment 1: shock-probe defensive burying test

CAPS treatment was initiated between PD 51–53 ($n$ = 9/group). One day following the end of CAPS (or the comparable time for controls), rats were tested in the shock probe defensive burying test to evaluate potential shifts in active and passive behavioral coping strategies in response to acute stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. Burying was defined as behavior consisting of burrowing into the bedding with the snout and upper body, then plowing, pushing, or shoveling the bedding toward the probe, and also flicking or spraying bedding material toward the probe. Immobility was defined as a lack of movement other than that required for breathing (slight scanning movements of the head were permitted). Behavior clearly identified as resting behavior (e.g., laying on side, legs extended) was excluded from immobility measures. As a proportional measure of preferred response, the bury time ratio was calculated as (time spent burying)/(time spent burying + time spent immobile).

### 2.4. Experiment 2: novelty suppressed feeding test (NSFT)

CAPS was initiated on PD 47 ($n$ = 14–15/group). Following CAPS (or the comparable time period for controls), the rats were left undisturbed for 2 days. Beginning on the 3rd day, the animals were food deprived for 48 h (water was available *ad libitum*). The test was conducted on the 5th day post-CAPS, as described by Bodnoff et al. (1988), with minor modification. The rats were transferred to the behavior room and allowed 1 h to acclimate. The rats were then individually placed into a corner of an unfamiliar black Plexiglas open field (100 × 100 × 40 cm) facing the center where food pellets were placed. The latency to begin feeding and the amount of food consumed during the 12 min test were recorded. Latency to feed was defined as the time from when the rats were placed into the open field until they began to eat the pellets (not just approach or play with them). Following the test, the rats were returned to their home cage, where food consumption was monitored for another 30 min to determine if there were any changes in appetitive behavior. Food consumption was determined by subtracting the weight of any remaining food from the total weight of food placed in the open field and the home cage.

### 2.5. Experiment 3: evoked HPA responses to acute immobilization stress

CAPS was initiated between PD 46–60 ($n$ = 7–14/group). Group assignments were matched to balance the range of ages at which CAPS was initiated across all groups. Three days prior to the acute prolonged stress (Day 12 of CAPS or the comparable time for controls), all rats underwent jugular catheterization surgery. Rats were anesthetized with a mixture of ketamine 43 mg/ml, acepromazine 1.4 mg/ml, xylazine 8.6 mg/ml, administered i.m. at 1.0 ml/kg, and a catheter comprised of silastic and PE50 tubing was inserted into the jugular vein, then passed subcutaneously and exteriorized via an incision at the back of the neck and plugged. Every

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3rd day until testing the catheter was flushed with approximately 0.2 ml of sterile heparinized saline (50 IU/ml) to maintain patency.

Separate groups were tested 1 or 5 days following the termination of CAPS. On the test day, the rats were transported to a quiet room, the catheter was connected via a fluid filled line to a syringe for remote blood collection without disturbing the animal, and approximately 0.1 ml of heparinized saline was administered to ensure patency. The rats were then given 90 min to acclimatize after transport. For blood sampling, 0.4 ml of blood was withdrawn via the catheter and replaced with 0.4 ml of sterile saline. Two baseline blood samples were collected 15 min apart. The rats were then immobilized for 30 min as described in Section 2.2. Two blood samples were collected during the stress, one at 5 min after the onset of stress, and one at 30 min. Following the 30 min stress sample, the rats were returned to their home cages and allowed to recover, during which time 4 blood samples were collected at 15, 30, 60, and 90 min post-stress. Blood was collected into tubes containing 10 μl of 0.5 M EDTA. Plasma was separated immediately by centrifugation at 10,000 rpm for 15 min at 4 °C, and stored at −80 °C until assayed.

Because the CAPS protocol includes a single 30-min immobilization stress, it was possible that any changes observed during the acute stress exposure on test day could be due to habituation or sensitization to the second presentation of immobilization stress, rather than an effect of CAPS specifically. Therefore, a control experiment was conducted in which 2 separate groups of rats ($n = 8–10$) were exposed to a single 30 min immobilization stress rather than the full CAPS procedure, 3 days after catheterization surgery. A third group was briefly handled but not immobilized. Then, 1 or 5 days later, all rats were exposed to an acute 30-min test immobilization, and blood samples were collected as above.

Plasma levels of ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100 μl samples according to the manufacturer's instructions (MP Biomedicals, Orangeburg, NY). The detection limit was 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to the manufacturer's instructions (MP Biomedicals). Detection limit was 8 ng/ml, and inter-assay variability was 8%.

### 2.6. Statistical analyses

For the shock-probe defensive burying data, differences in immobility time, active burying time, and bury-time ratio were analyzed by *t*-test. Likewise, in the novelty suppressed feeding test, differences between groups in latency to feed and amount of food consumed were analyzed by *t*-test. Plasma hormone measures were analyzed by 2-way analysis of variance (ANOVA; group × sample, with repeated measures). In all analyses, significance was determined at $p < 0.05$. After ANOVA, sources of any significant main effects or interactions were determined by analysis with the Newman–Keuls *post-hoc* test.

## 3. Results

### 3.1. Experiment 1: shock-probe defensive burying test

CAPS-treated rats displayed significantly less burying behavior than control rats (Fig. 1A, $t_{(16)} = 2.258$, $p < 0.05$) and significantly more immobility (Fig. 1B, $t_{(16)} = 2.963$, $p < 0.01$). Consequently, CAPS-treated rats displayed a significantly lower bury-time ratio than control rats (Fig. 1C, $t_{(16)} = 4.169$, $p < 0.001$). This reduction in bury ratio reflects a shift from a predominantly active behavioral coping strategy to a predominantly passive coping strategy.

### 3.2. Experiment 2: novelty suppressed feeding test

CAPS-treated rats displayed a significantly longer latency to feed (Fig. 2A, $t_{(27)} = 2.532$, $p < 0.05$). CAPS reduced weight gain during the treatment, as these rats had lower mean body weight (281.5 ± 5.3 g) than controls (298.0 ± 5.6 g) prior to testing (Fig. 2B, $t_{(27)} = 2.135$, $p < 0.05$), as expected after chronic cold stress. Nonetheless, there was no difference in the amount of food consumed during the test period (Fig. 2C, $t_{(27)} = 1.54$, $p > 0.05$), and CAPS-treated rats consumed slightly more food than controls in their home cage (Fig. 2D, $t_{(27)} = 2.451$, $p < 0.05$). The fact that CAPS-treated rats consumed equivalent amounts of food during the test, and more food than controls in the home cage indicates that the increase in latency to feed was not due to a reduction in appetite, but to an increase in anxiety in the novel environment.

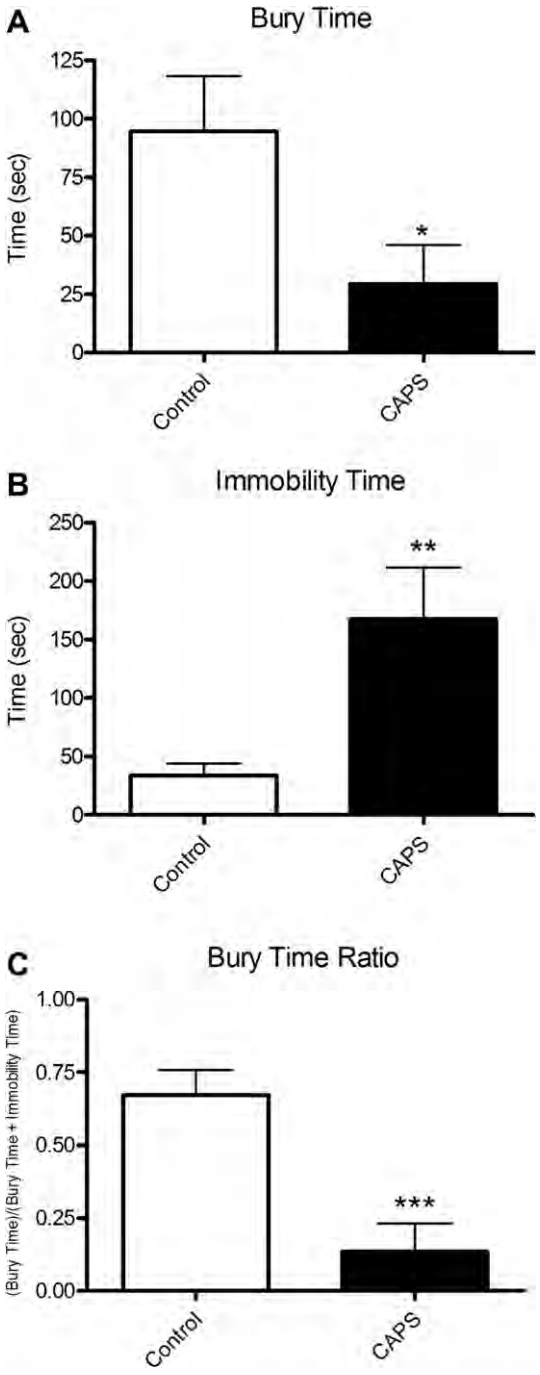


**Fig. 1.** Effect of CAPS on active defensive burying behavior and passive immobility in the shock-probe defensive burying test. A) On the shock-probe test, CAPS-treated rats displayed significantly less active burying behavior (29.33 ± 16.77 s) than unstressed control rats (94.67 ± 23.58 s). B) CAPS-treated rats displayed significantly more immobility (167.60 ± 43.98 s) in response to a single, brief mild shock than did the non-stressed controls (33.67 ± 10.39 s). C) Consequently, CAPS-treated rats had a lower bury ratio (0.13 ± 0.10) than control rats (0.67 ± 0.09), reflecting a shift from a predominantly active behavioral coping strategy (ratio > 0.5) in the control group to a predominantly passive strategy (ratio < 0.5) following CAPS treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data expressed as mean ± SEM, $n = 9$/group.

### 3.3. Experiment 3: evoked HPA response to acute immobilization stress

Acute immobilization stress induced a significant increase in both ACTH and CORT ($F_{(7,168)} = 47.8$, $p < 0.0001$; $F_{(7,182)} = 62.05$, $p < 0.0001$, respectively; significance not indicated in Fig. 3 for

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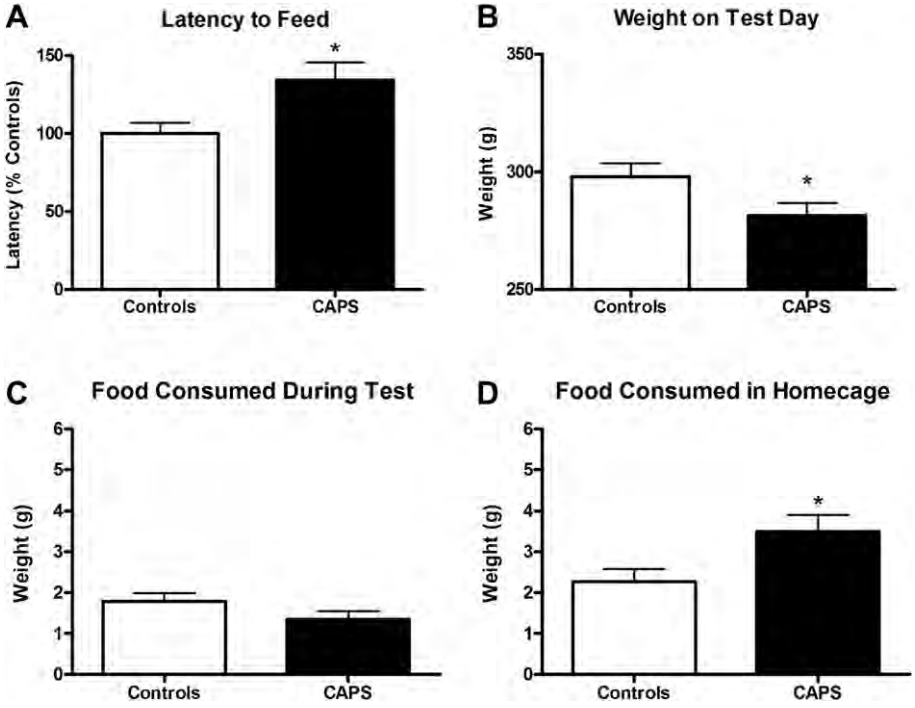


**Fig. 2.** Effect of CAPS on latency to feed in the novelty suppressed feeding test. A) CAPS-treated rats displayed a longer latency to begin eating (134.3 ± 11.4 % of controls, compared to 100.0 ± 6.9 %). B) As expected, CAPS-treated rats had lower mean body weight on test day (281.5 ± 5.3 g) compared to controls (298.0 ± 5.6 g). C) CAPS-treated rats and control rats consumed an equivalent amount of food during the test (1.35 ± 0.20 g and 1.79 ± 0.20 g, respectively). D) In fact, CAPS-treated rats displayed increased food consumption in the home cage after the test (3.5 ± 0.4 g) compared to control rats (2.3 ± 0.3 g), perhaps related to the reduction in body weight gain during CAPS treatment (panel B). Thus, the increase in latency to feed was not attributable to a loss of appetite, but to an increase in anxiety in the novel environment. $^{*}p < 0.05$. Latency expressed as mean percent of controls ± SEM. Feeding expressed as mean ± SEM, $n = 14$–15/group.

clarity). There was a significant main effect of Group on ACTH release (Fig. 3A, $F_{(2,24)} = 3.469$, $p < 0.05$) and a Group × Sample interaction ($F_{(14,168)} = 2.345$, $p < 0.01$). Specifically, CAPS-treated rats displayed a blunted ACTH response to acute stress. Post-hoc analyses revealed that the 1-day post-CAPS group was significantly different from the control group on the 5- and 30-min stress samples, and significantly different from the 5-day post-CAPS group on the 30-min stress sample. The effect on the 5-min stress sample for the 5-day post-CAPS group approached significance ($p = 0.0505$). Thus, the most robust effect of CAPS on the subsequent ACTH response to an acute immobilization stress occurred one day after the CAPS procedure, and by 5 days, the ACTH response was returning toward that seen in control rats. Although CORT levels in the 1-day post-CAPS group were slightly but consistently lower than in controls, this was not a significant reduction (Fig. 3B, $F_{(2,26)} = 1.542$, $p > 0.05$ for main effect of stress; $F_{(14,182)} = 1.228$, $p > 0.05$ for stress × sample interaction).

The control experiment, conducted to ensure that the blunted ACTH response in CAPS-treated rats was not due simply to habituation to a second exposure to immobilization stress, showed no effect of prior immobilization on ACTH release in response to the test immobilization stress (Fig. 4, $F_{(2,24)} = 1.162$, $p > 0.05$), nor was there a group × sample interaction ($F_{(14,168)} = 0.871$, $p > 0.05$). Because the effect of CAPS on the ACTH response in experiment 3 was seen only at 1-day post-CAPS, we also analyzed the results of the control experiment for day 1 only, excluding day 5. There was still no effect of prior immobilization ($F_{(1,17)} = 1.161$, $p > 0.05$) nor an interaction ($F_{(7,119)} = 1.224$, $p > 0.05$). Thus, these results suggest that the changes in evoked ACTH response following exposure to CAPS were due to the CAPS treatment specifically, and not due simply to a second exposure to immobilization. Because the effect of CAPS on CORT did not achieve significance in experiment 3, CORT was not analyzed in the control experiment.

## 4. Discussion

In the present set of studies, CAPS-treated rats displayed a shift from an active to a passive coping style, and an increase in anxiety-related behavior. These behavioral effects co-occurred with a blunted ACTH response to acute stress. In addition to these changes, we have previously reported that CAPS impaired fear extinction and reduced GR expression in the mPFC (Green et al., 2011).

### 4.1. Passive coping

In our previous report (Green et al., 2011), prenatal stress did not alter the preference for active coping relative to passive coping on the shock-probe defensive burying test (prior to CAPS exposure). However, after CAPS exposure, we noted a different behavioral profile during fear conditioning. After multiple tone–shock pairings, control rats begin to show a decrease in freezing, which appears to be due to a shift in behavioral response to the tone, away from the passive freezing response to more active escape behaviors, including rearing and jumping. In that study, rats exposed to prenatal stress and CAPS as adults did not display this shift. Rather, they continued to display high levels of freezing. While this observation is anecdotal, and behavior observed during fear-conditioning is not a validated measure of coping style, this led us to hypothesize that CAPS might produce a shift from active coping to a more passive coping strategy. This hypothesis was tested explicitly in the present study using the shock probe defensive burying test, in which rats can exhibit 2 qualitatively different types of behavioral responses to the shock probe in varying proportions—an active response (burying the probe) and a passive response (immobility). Control rats displayed a slight preference for active coping behavior (burying), whereas CAPS-treated rats displayed a substantial shift to a strong preference for

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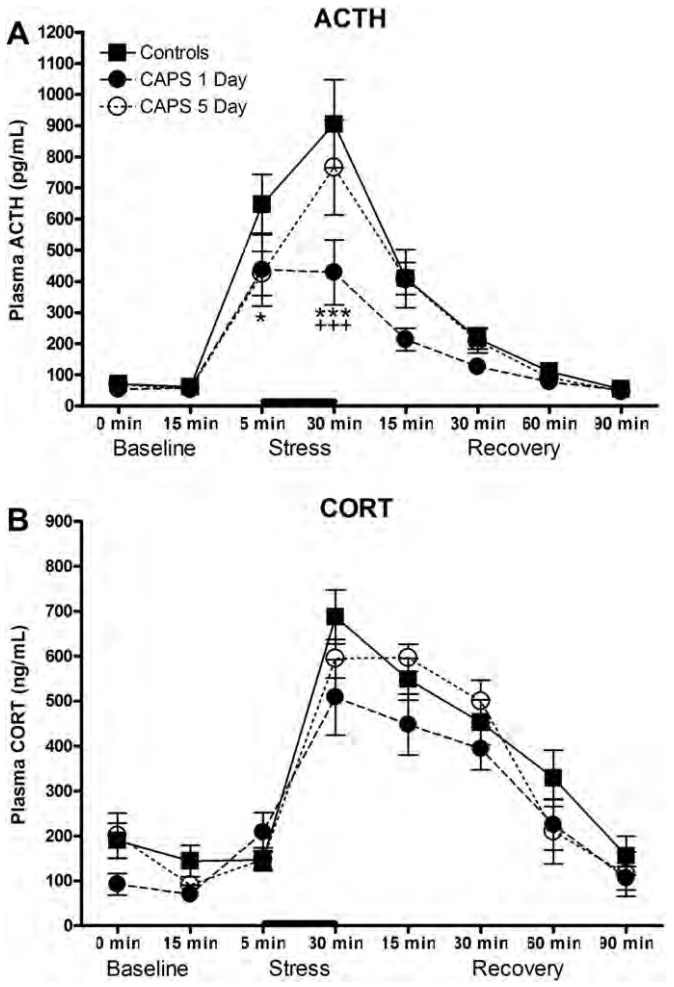


**Fig. 3.** Effect of CAPS on HPA stress reactivity in response to acute immobilization stress. A) CAPS-treated rats displayed a blunted ACTH response to a subsequent acute stressor (bar), particularly in the group tested on day 1 post-CAPS. B) There were no significant differences between groups on the acute CORT response to immobilization stress, although there was a modest decrease in the group tested on day 1 post-CAPS. $^{*}p < 0.05$ 1-day post-CAPS vs controls, $^{***}p < 0.001$ 1-day post-CAPS vs controls, $^{+++}p < 0.001$ 1-day post-CAPS vs 5-days post-CAPS. Data expressed as mean ± SEM, $n = 7–14$/group.

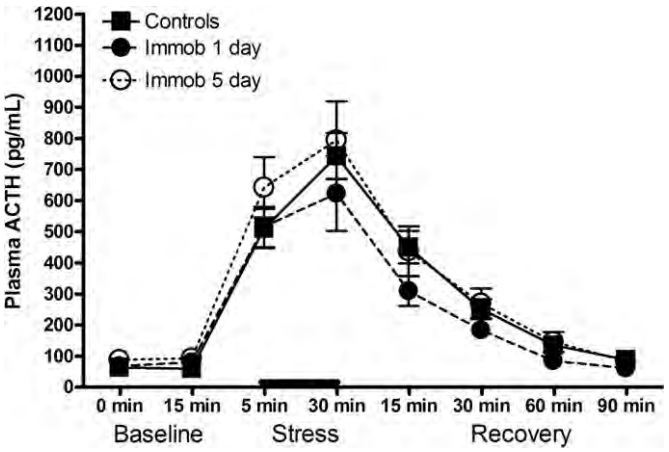


**Fig. 4.** Lack of effect of a single prior exposure to acute immobilization stress on the subsequent ACTH response to a second immobilization stress. There was no effect of prior immobilization on ACTH release evoked by a second immobilization stress (bar) administered either 1 or 5 days later, suggesting that the effect seen in Fig. 3 was due to CAPS exposure, specifically. Data expressed as mean ± SEM, $n = 8–10$/group.

passive coping behavior (immobility). The increase in immobility cannot be explained by an overall decrease in locomotor activity, as we previously showed that CAPS-treated rats displayed no change in exploration in an open field (Green et al., 2011).

Coping style can mitigate the physiological impact of stress, and there is evidence from both animal and human research that active coping is more adaptive. Previous research has shown that when a rat is given the option to engage in an active coping response, such as chewing on a dowel during immobilization, the stress response is reduced (e.g., Hori et al., 2004; Ono et al., 2008). By contrast, when a rat is deprived of an active response option, such as removing the bedding during the shock probe test so the rat cannot bury, the physiological stress response is increased (Bondi et al., 2007). Likewise, rats that show a low bury response have higher CORT responses during the test (for review see Koolhaas et al., 1999).

A shift to immobility in the shock probe test resembles the "learned helplessness" phenomenon described in both the human and animal psychological literature. Passive responding and failure to engage in active coping responses has long been demonstrated in a number of animal models, originally in dogs (e.g., Seligman et al., 1968) and then rodents (e.g., Maier, 1984). The learned helplessness model shares some characteristics with the CAPS model. For example, rodents exposed to inescapable tail shock display less aggression in a shock-elicited aggression test (similar to our finding of reduced active burying in the shock-probe defensive burying test) and reduced intruder attack by alpha males (Maier, 1984). These rats also display cognitive deficits, including more errors in tests involving learning contingencies (Maier, 1984) and delayed contextual fear extinction (Baratta et al., 2007). Again, this is similar to our previous finding that CAPS treatment impaired fear extinction.

Passivity may contribute to maladaptive stress responses. Consistent with this, studies with animals and humans have shown that active, stimulus-based, or problem-oriented coping styles, as opposed to more passive, emotion-based, avoidant coping styles, buffer HPA activation in response to the stressor/stimulus, increase the ability to eliminate the threat, and improve long-term mental and physical health outcomes (for review see, Koolhaas et al., 1999; Olff et al., 2005). On the other hand, in humans, the negative thought patterns related to many affective disorders often contribute to the perception that there is no way out of a stressful situation and little or no control over one's situation and environment. Emotional withdrawal is a key symptom in the diagnosis of PTSD (American Psychiatric Association, 2000), and studies have shown that individuals previously exposed to traumatic events show greater levels of introversion, social isolation, and emotional blunting (e.g., Bunce et al., 1995). A predisposition for withdrawal may actually contribute to the development of PTSD, and greater symptom expression over time. For example, traumatized individuals who show a shift toward passive coping styles, including withdrawal, are more likely to develop PTSD at 3 months post-trauma (Gutner et al., 2006). Furthermore, men who were abused as children and display high levels of introversion and withdrawal are more likely to meet thresholds for clinical diagnosis of PTSD in adulthood (O'Leary, 2009). Similarly, individuals who report peri-traumatic feelings of helplessness are more likely to develop PTSD (Beck et al., 2006; Hari et al., 2010; O'Donnell et al., 2010).

### 4.2. Anxiety

We previously examined the effect of CAPS on anxiety-related behavior in an open field and found no differences. In the present experiment, we examined potential anxiogenic effects of CAPS

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using a more robust test of anxiety involving an approach-avoidance conflict, the novelty suppressed feeding test. In this test, food deprived rats must enter an anxiety-provoking environment to obtain food. Previous studies with the NSFT have shown that chronic stress results in longer latency to approach the food and begin eating, particularly in more passive, "low responder" rats (Stedenfeld et al., 2011), and that antidepressant treatment reduces the expression of anxiety-related behaviors in this test (Furmaga et al., 2011; Ibarguen-Vargas et al., 2009). Similarly, CAPS-treated rats displayed an increased latency to feed in the novel environment, reflecting greater anxiety, despite an increase in total food intake.

Anxiety is an important component of most animal models of human stress-related psychiatric disorders, as anxiety is a key element of such disorders, including PTSD. Further, PTSD is highly comorbid with other anxiety disorders, and also with depression (Kessler et al., 1995; Rush et al., 2005). These disorders are all notable for an extensive degree of overlap in symptomatology, including, for example, irritable mood, difficulty concentrating, and sleep disturbances. Further, antidepressants are also effective pharmacological treatment for many anxiety disorders (for review, see Morilak and Frazer, 2004). Thus, there are likely to be common neurobiological mechanisms and similar psychopathological processes underlying these shared symptoms.

### 4.3. Acute HPA stress-reactivity

CAPS treatment reduced the acute ACTH stress response, especially on day 1 after the termination of CAPS. The control experiment confirmed that this was not merely due to habituation to the prior exposure to immobilization stress on day 15 of CAPS treatment. There was also a slight but non-significant suppression of the CORT response to acute stress, and during the post-stress recovery period. It is not clear why the effect of CAPS on the acute CORT response was less robust than on the ACTH response. It may simply be due to differences in the temporal sensitivity of these measures. ACTH is rapidly and dynamically reactive. However, with CORT being slower to respond and slower to clear, a sample at any given time point represents a cumulative response. Thus, differences may have been obscured. On the other hand, it is possible that the adrenal glands may have been sensitized by the previous stress exposure, resulting in greater CORT release in response to ACTH, thus compensating in part for the reduction in evoked ACTH levels. Previous research has shown that chronic stress increases adrenal mass, which may contribute to such sensitization (e.g., Blanchard et al., 1998; Hauger et al., 1990). Another possibility may be related to intensity of the stress induced by immobilization. The HPA response to immobilization was very robust, and may have masked a modest difference between CORT responses in Control and CAPS-treated rats. It may be informative to employ a milder probe stimulus in future studies. Finally, it is important to note that the baseline CORT levels in this experiment were higher than those reported in our previous study (Green et al., 2011). This is likely attributable to differences in methodology. In the present study, rats were exposed to surgery, and then on the test day to handling and a novel environment, all of which can elevate baseline CORT levels, even with a period of acclimation. In the previous study, CORT levels were measured in trunk blood samples collected by rapid decapitation immediately after removal from their home cages.

The changes observed in acute HPA axis stress reactivity are interesting in light of the human PTSD literature. Evidence suggests that HPA activity is reduced in PTSD. However, the full HPA axis profile of individuals with PTSD is not clear, and there are many inconsistencies in the literature (for discussion, see de Kloet et al., 2006). Some studies have shown urinary and plasma cortisol levels to be lower in PTSD patients compared to controls (Boscarino, 1996; Mason et al., 1986; Yehuda et al., 1995, 1993, 1990), and these hormone levels may be negatively correlated with symptom severity (Olff et al., 2006). Further, individuals with lower CORT levels at the time of the post-trauma emergency room visit are more likely to develop PTSD (Delahanty and Nugent, 2006). Reduced hormonal responses may be due to a sensitized negative feedback mechanism, as PTSD patients tend to show greater ACTH suppression by dexamethasone (e.g., Duval et al., 2004). The present results are in line with these findings.

Few animal models of stress have replicated the HPA-axis characteristics of human PTSD, as the typical effect of chronic stress exposure in rodent models is sensitization of the HPA response to acute stress, if any change is observed at all. Rimanoczy et al. (2003) showed that prenatal stress exposure to morphine resulted in a suppressed ACTH response to restraint in adulthood, while maintaining a normal CORT response, similar to the effect seen in our study. Similarly, the SPS model, from which the acute component of our CAPS model was adapted, enhanced HPA suppression in response to dexamethasone treatment (Yamamoto et al., 2009). Further, rats exposed to SPS also display a blunted CORT response to a subsequent acute stressor (Harvey et al., 2006).

By comparison, varying alterations in ACTH and/or CORT have been reported in studies employing the widely-used Chronic Variable Stress (CVS)/Chronic Mild Stress (CMS) model. Most have shown either no change or an increase in basal ACTH (e.g., Choi et al., 2008a,b; Kioukia-Fougia et al., 2002; Ostrander et al., 2006) and no change or an increase in basal CORT (e.g., Choi et al., 2008a,b; Christiansen et al., 2012; Ostrander et al., 2006; Wu and Wang, 2010). Blunting of circadian cycles has been reported (Christiansen et al., 2012). Changes in HPA response to acute stress challenge after CVS/CMS are variable. One study reported an increased ACTH response to acute restraint stress, but no change in CORT response (Choi et al., 2008b). In another, an increase in ACTH response to a mild novelty stress was seen 1 day after CVS, which returned to normal on day 4 post-CVS, followed by a decrease in ACTH response on day 7, returning to baseline by day 30 (Ostrander et al., 2006). As in the present study (and in Choi et al., 2008b), the CORT response did not match the ACTH response. There was no change in the CORT response one day post-CVS, a decrease at days 4 and 7, then a return to normal by day 30. By contrast, when this same group challenged with a systemic stressor (hypoxia), the effect was similar to that seen in the present study, a decrease in ACTH response one day post-CVS, which returned to normal on day 4, with no change in CORT. ACTH then increased on day 7 post-CVS, again with no comparable change in CORT. Other factors that can affect changes in hormonal response after stress are anhedonia-like traits (Christiansen et al., 2012) and strain differences (Wu and Wang, 2010). In most chronic stress models, regardless of the nature of the change in HPA response, it is important to note that, as in the present study, effects were transient, and changes in ACTH and CORT responses are often dissociated.

These results would suggest that an HPA regulatory process that blunts the ACTH response to a subsequent acute stressor emerges in response to chronic stress, then dissipates over time when the stress ceases. In humans with PTSD, even after termination of the primary stressor, the cognitive process of re-experiencing may become a secondary chronic stressor on its own, maintaining the dysregulatory process. Thus, animal models may be particularly useful in revealing mechanisms by which pathological processes after traumatic stress are initiated, and in identifying unique mechanisms by which HPA responses may be inhibited in PTSD, as opposed to the hyperactive HPA axis often seen in other chronic stress-related mood disorders, such as depression.

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Despite the transient effect of CAPS on the HPA response to acute stress, behavioral effects were evident at all time points. We examined the effects of CAPS on shock-probe defensive burying behavior on day 1 post-stress, comparable to when we observed the greatest ACTH suppression. However, the need for food restriction in the novelty-suppressed feeding test, and the desire to avoid confounding stress with food deprivation, necessitated testing on day 5 post-stress. In both cases, at 1 day and 5 days post-stress, we observed behavioral effects of CAPS treatment. In general, then, it appears that although the HPA effects begin to recover by day 5, the behavioral effects are evident at day 1 (increased passive coping in shock probe defensive burying), day 2 (increased freezing in fear conditioning, Green et al., 2011), and still present at day 5 (increased anxiety in NSFT, and impaired fear extinction, Green et al., 2011).

### 4.4. Conclusion

Valid animal models of human psychopathology must be based on a theoretical framework that shares a fundamental aspect of the human disorder, and they must show behavioral and biochemical features that resemble those in the human disorder (Willner, 1986). One requirement for a diagnosis of PTSD is experience of a traumatic event (American Psychiatric Association, 2000). This was modeled by the CAPS treatment in the present study, involving a low-level chronic "state" of stress, followed by a highly salient and intense acute stress experience, which may model the kinds of experiences that initiate PTSD, particularly in combat veterans. Chronic stress is correlated with vulnerability to PTSD (Breslau et al., 1999; Koenen et al., 2007, 2002), and in combat situations, chronic stress, punctuated by acute traumatic events, is the norm. Further, once the trauma has been experienced, an exaggerated and persistent fear response is arguably the fundamental aspect of PTSD (American Psychiatric Association, 2000), and this may be prolonged by impairments in extinction learning (Blechert et al., 2007; Wessa and Flor, 2007; Jovanovic et al., 2009). In our previous report, we showed that CAPS exposure enhanced freezing during fear conditioning and impaired extinction. Further, in the present study, CAPS resulted in other PTSD-like symptoms, including: anxiety; a shift from effective active coping to less adaptive passive coping; and HPA axis dysregulation.

The brain mechanisms that underlie these effects remain to be elucidated. CAPS is a combination of chronic metabolic stress (chronic cold) and a single session of intense acute stress that was adapted from the single-prolonged stress model (SPS; Yamamoto et al., 2009, 2010). Each of these components may have neurobiological consequences that contribute to the resulting phenotype. SPS has been shown to increase inhibitory avoidance, decrease extinction, and increase acoustic startle (Yamamoto et al., 2010; Ganon-Elazar and Akirav, 2012; Knox et al., 2012). This may be due, in part, to reduced excitatory neurotransmitter tone in the PFC and hippocampus, as SPS decreased glutamate and creatine in the PFC, and increased glycine transporter expression in the ventral hippocampus (Yamamoto et al., 2010; Knox et al., 2010). Chronic cold has been shown to impair cognitive flexibility and to decrease serotonin release in the orbital frontal cortex (Lapiz-Bluhm et al., 2009). Changes in prefrontal executive function could compromise the ability to regulate or select from among possible responses in fear- or anxiety-provoking situations. Chronic cold stress alone has been shown to sensitize the ACTH response to immobilization stress (Ma and Morilak, 2005), whereas SPS increased negative feedback inhibition of ACTH release (Liberzon et al., 1997), similar to the blunted ACTH response in the present study. Thus, the phenotype of CAPS-treated rats appears to be a combination of acute and chronic stress effects, perhaps involving changes in modulatory neurotransmission in the prefrontal cortex, consistent with our previous observations of altered GR expression following CAPS treatment (Green et al., 2011). This further suggests that drugs that modulate monoaminergic transmission, glucocorticoid activity, or excitatory amino acid signaling may represent viable strategies for treatment and symptom management of PTSD. Interestingly, it was recently reported that the SPS-induced increase in glycine transporter expression in the hippocampus was normalized with repeated extinction training, perhaps identifying a mechanism by which therapeutically effective behavioral interventions can also mitigate the effects of chronic stress (Yamamoto et al., 2010).

In sum, the CAPS model may prove useful as a valid animal model with which to investigate neurobiological mechanisms underlying pathophysiological changes associated with PTSD, or mechanisms of novel therapeutic strategies for PTSD.

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# Exogenous prenatal corticosterone exposure mimics the effects of prenatal stress on adult brain stress response systems and fear extinction behavior

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**Summary** Exposure to early-life stress is a risk factor for the development of cognitive and emotional disorders later in life. We previously demonstrated that prenatal stress (PNS) in rats results in long-term, stable changes in central stress-response systems and impairs the ability to extinguish conditioned fear responding, a component of post-traumatic stress disorder (PTSD). Maternal corticosterone (CORT), released during prenatal stress, is a possible mediator of these effects. The purpose of the present study was to investigate whether fetal exposure to CORT at levels induced by PNS is sufficient to alter the development of adult stress neurobiology and fear extinction behavior. Pregnant dams were subject to either PNS (60 min immobilization/day from ED 14—21) or a daily injection of CORT (10 mg/kg), which approximated both fetal and maternal plasma CORT levels elicited during PNS. Control dams were given injections of oil vehicle. Male offspring were allowed to grow to adulthood undisturbed, at which point they were sacrificed and the medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and a section of the rostral pons containing the locus coeruleus (LC) were dissected. PNS and prenatal CORT treatment decreased glucocorticoid receptor protein levels in the mPFC, hippocampus, and hypothalamus when compared to control offspring. Both treatments also decreased tyrosine hydroxylase levels in the LC. Finally, the effect of prenatal CORT exposure on fear extinction behavior was examined following chronic stress. Prenatal CORT impaired both acquisition and recall of cue-conditioned fear extinction. This effect was additive to the impairment induced by previous chronic stress. Thus, these data suggest that fetal exposure to high levels of maternal CORT is responsible for

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many of the lasting neurobiological consequences of PNS as they relate to the processes underlying extinction of learned fear. The data further suggest that adverse prenatal environments constitute a risk factor for PTSD-like symptomatology, especially when combined with chronic stressors later in life.
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## 1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling affective disorder that occurs as a consequence of a physically or emotionally traumatic experience. It is characterized by intrusive memories, a state of hyper-vigilance, and an inability to inhibit fear responses to trauma-associated cues. An estimated 9 million people in the United States suffer from PTSD, yet this is only a portion of those who experience trauma (Kessler et al., 2005). Therefore, other factors are likely to confer vulnerability to developing PTSD subsequent to traumatic stress, including experiential, environmental, or biological predispositions. Clinical studies suggest that early life stressors, such as childhood exposure to trauma, low socioeconomic status, and familial instability increase susceptibility to PTSD later in life (Breslau et al., 1999; Koenen et al., 2007). Prenatal stress (PNS) is an adverse early life event that has been associated with increased risk for anxiety, ADHD, schizophrenia, developmental delays, and hypothalamic–pituitary–adrenal (HPA) axis dysregulation in humans; however, very little is known about its role as a potential risk factor for PTSD (Davis and Sandman, 2012; Talge et al., 2007).

Animal studies suggest that PNS programs the adult stress system to create a stress-reactive phenotype, showing fear- and depression-like behaviors that resemble aspects of PTSD (Weinstock, 2008). PNS has been shown to permanently program the brain corticosteroid and brain monoamine systems, both of which are implicated in the formation and extinction of fear memories. PNS can reduce glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR) expression in adult offspring (Brunton and Russell, 2010; Green et al., 2011; Harris and Seckl, 2011; Weinstock, 2008). PNS also alters catecholamine release in brain areas associated with behavioral and cognitive components of the stress response. It has been shown to decrease basal and stress-induced norepinephrine release in the prefrontal cortex (PFC) and locus coeruleus (LC) as well as dopamine in the LC (Carboni et al., 2010; Takahashi et al., 1992). In addition to these biochemical effects, PNS causes enduring behavioral changes, including anxiety-like behavior on the elevated plus maze, an increase in freezing behavior following footshock, and compromised performance in cognitive tasks like the Morris water maze (Brunton and Russell, 2010; Kofman, 2002; Salomon et al., 2011; Takahashi et al., 1992; Weinstock, 2008).

This altered physiological and behavioral response to stress may create a state of vulnerability to chronic stressors later in life and thus increase the risk for PTSD. Indeed, after experiencing a traumatic stress, many individuals continue to endure a secondary, persistent state of chronic stress that is produced by intrusive memories, nightmares, and increased physiological stress responses to cues associated with the initial trauma. In individuals who may be impaired in their ability to cope with stress, this persistent chronic stress may facilitate the transition from acute stress disorder to chronic PTSD (Davidson and Baum, 1986; Wessa and Flor, 2007). Likewise, chronic stress may also impair the ability to extinguish trauma-associated fear memories. Preclinical studies in rodents have demonstrated that chronic stress in adulthood facilitates conditioned fear behavior and impairs the retention of subsequent extinction of conditioned fear (Farrell et al., 2010; Garcia et al., 2008). We recently showed that both PNS and adult chronic stress independently impaired acquisition of fear-extinction. These effects appeared to be additive, such that rats receiving both PNS and adult chronic stress were consistently the most impaired in their ability to extinguish fearful associations, a hallmark trait of PTSD in humans (Green et al., 2011).

Fetal exposure to maternal glucocorticoids represents one potential mechanism whereby PNS may program the adult stress response in utero. During PNS, glucocorticoids are released by the dam, and, at high concentration, can cross the placental barrier to exert direct effects on gene transcription in the fetus (Harris and Seckl, 2011; Takahashi et al., 1998). Fetal exposure to high levels of glucocorticoids results in long-term impairments in cognitive and emotional regulation (Alexander et al., 2012). Both the direct administration of glucocorticoids and the inhibition of placental barrier enzymes mimic some of the effects of PNS (Welberg et al., 2000). Likewise, maternal adrenalectomy is able to prevent some of the lasting effects of PNS (Barbazanges et al., 1996; Salomon et al., 2011). However, it is unknown if prenatal glucocorticoid exposure mimics the effects of PNS on the formation and extinction of fear memories in the adult offspring. It is also unknown whether a history of prenatal glucocorticoid exposure interacts with later stress to further impair fear extinction, i.e., creating vulnerability for a PTSD-like phenotype. To address these questions, we compared the effects of prenatal corticosterone (CORT) administration in the absence of maternal stress to those of PNS. We first determined a dose of exogenous CORT, delivered to the mother, that best mimics both fetal and maternal circulating CORT levels induced by PNS. To determine if CORT treatment mimics the neurobiological consequences of PNS, we then measured the mRNA and protein expression of the GR, corticotrophin releasing factor (CRF), brain-derived neurotrophic factor (BDNF), and tyrosine-hydroxylase (TH) in the brains of the adult male offspring. Finally, we measured the effect of prenatal CORT exposure on fear conditioning and extinction behavior in the adult offspring, with and without exposure to chronic stress. We hypothesized that prenatal CORT exposure would mimic the neurobiological effects of PNS and create an additive detrimental effect on fear conditioning and extinction behavior when combined with chronic stress later in life.

## 2. Methods

### 2.1. Animals

Timed-pregnant female Sprague-Dawley rats (Harlan, Indianapolis) arrived on embryonic day (ED) 6 and were

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single-housed in standard Plexiglas cages (25 cm × 45 cm × 15 cm) on a 12/12 h light–dark cycle (lights on at 7:00 h) with food and water available ad libitum. On postnatal day (PD) 5, litters were culled to eight pups each, maximizing the number of males. Upon weaning (PD 21), male pups were housed 2–3 per cage with littermates until PD 45, at which time they were single-housed. In total, 154 adult male offspring (from 30 litters: 11 stressed, 9 CORT-treated, 10 control) were used in these experiments. An additional 32 females were sacrificed at E16 or E20 to provide maternal and fetal CORT levels. For the social defeat procedure, 12 adult male Long-Evans rats (Harlan), weighing at least 400 g, were used as defeaters. Each resident male was housed in a large cage (80 cm × 55 cm × 40 cm) with an ovariectomized female, in a separate room from the experimental colony. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering, or discomfort, and to minimize the number of rats used.

## 2.2. Prenatal stress

From ED14 to ED21, stressed females were injected daily with either sesame oil vehicle (1.4 ml/kg, sc. Sigma–Aldrich) or saline vehicle (4.5 ml/kg, sc.), depending on the experiment. They were then immobilized for 60 min. They were held gently but firmly on a flat rack while the limbs, shoulders, and hips were taped to the rack. The midsection was not taped to avoid putting physical pressure on the fetuses. The animals were unable to move, but respiration was unhindered and they were not enclosed to avoid hyperthermia. Unstressed females were injected with either CORT (10 mg/kg, sc. Sigma–Aldrich), saline, or oil vehicle and returned to their home cages. This dose was established in pilot studies to approximate stress CORT levels.

## 2.3. Measurement of fetal corticosterone levels

Dams from each treatment group were sacrificed by rapid decapitation immediately after 1 h immobilization, or 1 h following CORT or vehicle injection on day ED16 or ED20. Maternal trunk blood was collected in chilled 15 ml conical tubes containing 100 μl of 0.5 M EDTA. Immediately thereafter (<1 min), pups were removed by Cesarean section and rapidly decapitated. For the E20 fetuses, approximately 70 μl of trunk blood was collected from each fetus ($n$ = 1–3/litter) with a heparinized capillary tube, and deposited into ice-cold eppendorf vials. Because of the small fetal blood volume at E16, each sample ($n$ = 1–2/litter) represents blood pooled from 3 sibling fetuses, approximately 70 μl total. After collection, blood samples were centrifuged at 4 °C (3000 × $g$ for 15 min) and the plasma removed and stored at −20 °C. Plasma CORT levels were determined via radioimmunoassay as previously described (Roth et al., 2012).

## 2.4. Chronic plus acute prolonged stress (CAPS)

To investigate the interaction between prenatal CORT exposure and behavioral susceptibility to chronic stress later in life, offspring in each treatment condition were subjected to chronic plus acute prolonged stress (CAPS) treatment from PD 46–48 to PD 60–62. The chronic component of the CAPS procedure entailed 14 days of chronic intermittent cold stress. The rats were transported in their home cage, with food, bedding, and water, into a cold room (4 °C, 6 h/day) for 14 consecutive days. The acute component on day 15 consisted of 3 acute stressors administered sequentially in a single 1-h session: social defeat (20 min), immobilization (30 min), and forced swim (10 min). For social defeat, the ovariectomized Long-Evans female was removed from the resident cage, and the test rat was placed into the resident cage. After the resident Long-Evans male rat attacked and defeated the test rat, defined by the test rat assuming a submissive posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, protecting it from further physical contact but allowing continued sensory interaction. Immobilization involved taping the torso, head, and limbs gently but firmly in a prone position on a flat platform, allowing no movement for 30 min. For swim stress, the rat was placed in a cylindrical tank (30 cm diameter × 60 cm height) filled to a depth of 30 cm with water at approximately 23 °C.

## 2.5. Fear conditioning and extinction

Fear conditioning and extinction were performed as previously described with minor modification (Green et al., 2011). One day after the termination of CAPS treatment (or the comparable time point for controls), rats were habituated to two contexts for 15 min each. Twenty-four hours after habituation, the rats received cued fear conditioning in Context A, a shock chamber with metal walls and a grid floor. Each rat was placed into the chamber and, after a 5 min acclimation period, experienced four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminating with a shock (0.8 mA, 0.5 s, average inter-trial interval = 120 s). Extinction training occurred 3 days later. The rats were placed in Context B, a similar chamber but with smooth vinyl floors and walls to avoid contextual freezing. They were exposed to 16 trials of the tone alone, with an average inter-trial interval of 2 min. On the following day, the rats were returned to Context B, and the retention of extinction was tested by presenting them with 16 additional tones. Behavior during each stage was video-recorded and freezing behavior during each tone presentation was analyzed off-line using the FreezeFrame and FreezeView software (Coulbourn Instruments #ACT-100). Freezing was defined as behavior below a motion index threshold of 10 lasting at least 1 s.

## 2.6. Tissue collection

Rats were sacrificed on PD 65–67 by rapid decapitation. The medial prefrontal cortex (mPFC), hippocampus, hypothalamus, and the pontine area containing the locus coeruleus (LC) were quickly dissected using a brain matrix on ice, as described previously (Green et al., 2011). The brain samples were frozen on dry ice and stored at −80 °C until use. TH mRNA and protein were analyzed in the LC samples; mRNA and protein for GR in mPFC, hippocampus, and hypothalamus samples; mRNA and protein for CRH in hypothalamus

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samples; and mRNA and protein for BDNF in hippocampus samples.

### 2.7. mRNA analyses

Total RNA was isolated from each brain region and converted to cDNA as described previously (Green et al., 2011). Real-time PCR was performed using the following Taqman gene expression assays (Applied Biosystems/Life Technologies, Carlsbad, CA): rat TH, Rn00562500_m1; rat GR (NR3C1), Rn00561369_m1; rat CRH, Rn01462137_m1; and rat BDNF, Rn01484924_m1. All assays consisted of intron-spanning primers and FAM-labeled probes. Results were normalized using the eukaryotic 18S rRNA endogenous control assay (4319413E) labeled with VIC dye. Assays were performed in triplicate after validating with the ABI Prism 7900 HT instrument and following the MIQE guidelines. Real time PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method. Relative expression of the gene of interest in treatment groups was expressed as percent of control.

### 2.8. Protein analyses

TH protein in the LC region was analyzed by Western blot as described previously (Green et al., 2011). GR protein was analyzed using an ELISA kit (TransAM GR kit, Active Motif, Carlsbad, CA) and protein levels were computed from A450 values. CRH protein levels in hypothalamus or hippocampus were assayed using an extraction-free Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA). Similarly, BDNF protein levels in the hippocampus were assayed using a rat BDNF ELISA kit (Syd Labs Inc., Malden, MA) following the manufacturer's protocol. All results were expressed as a percent of the oil-treated non-stressed control mean.

### 2.9. Data analysis and statistics

Maternal and fetal CORT measures were analyzed by ANOVA at each age, with Newman–Keuls Multiple Comparison post-tests where significant effects were revealed. All adult neurochemical measures were analyzed by ANOVA with Dunnett's post-test used for comparison to the vehicle control group. For the fear conditioning, extinction, and retention data, group differences in percent freezing were first analyzed for each session by a three-way ANOVA (prenatal CORT × adult stress × tone) with repeated measures over tone. In addition, total freezing during extinction, represented by the mean area under the extinction curve, was analyzed by 2-way ANOVA with Bonferroni post-tests for pairwise comparisons. Subsequently, to better assess and compare the rate and extent of extinction across groups, the freezing data for all animals within a group were best fit to an unconstrained, single-exponential decay function using Graphpad Prism 5. As reported previously (Green et al., 2011), freezing typically increased from tone 1 to tone 2 in the extinction session. Therefore, tone 1 was not included in the regression analysis, so that the extinction rate could be calculated from the point of maximum freezing. From the resulting regression equations, the decay constant ($k$), plateau value, and their standard errors (SE) were derived for each group. Differences between groups were analyzed using an extension of Cochran's Q methodology (Cochran, 1954) which partitioned the overall Chi-square (df = 3) into independent factor components according to a 2 (prenatal CORT exposure) × 2 (adult stress) design. The $Q$ statistics were then transformed to $F$ values as described (Cochran, 1954).

## 3. Results

### 3.1. Maternal and fetal corticosterone levels

Both PNS and CORT treatment significantly increased maternal CORT levels (Fig. 1A) measured at E16 ($F_{(3,15)} = 58.07$; $p < 0.01$) and E20 ($F_{(3,15)} = 60.89$; $p < 0.01$) when compared to the unstressed saline and oil-treated controls ($p < 0.01$). At E16, there were no differences in the plasma CORT levels of PNS dams compared to CORT-treated dams; however, at E20, the PNS-induced plasma CORT level was slightly higher than that of the CORT-treated dams ($p < 0.01$). There were no differences in CORT levels between saline- and oil-treated dams at either age. As with the dams, PNS and CORT treatment also increased fetal CORT levels (Fig. 1B) at E16 ($F_{(3,25)} = 18.71$; $p < 0.01$) and E20 ($F_{(3,39)} = 10.41$; $p < 0.01$) when compared to the respective unstressed saline- and oil-treated control groups at both ages ($p < 0.01$). There were no differences in fetal plasma CORT between saline- and oil-treated groups, or between PNS and CORT groups at either age. As expected, fetal CORT increased from

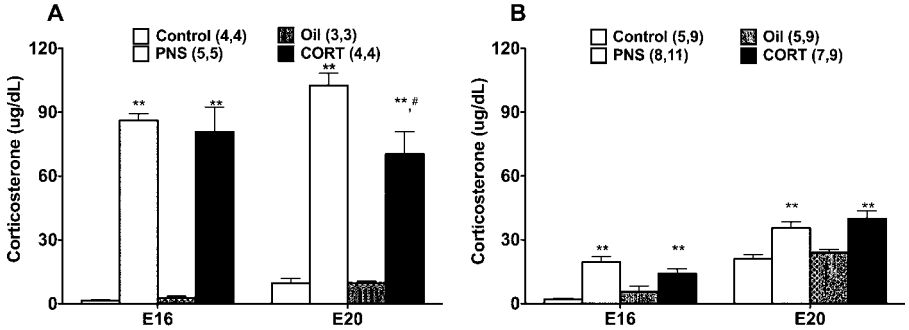


**Figure 1** Maternal (A) and fetal (B) plasma CORT levels following a 60 min immobilization stress or 60 min after acute CORT injection on embryonic day ED16 or ED20. Both PNS and CORT increased plasma CORT compared to the respective control treatments in both the dams and fetuses at each day (**$p < 0.01$). On E20, PNS induced a slightly larger increase than CORT treatment in the dams ($^{\#}p < 0.01$); however, there were no differences in the corresponding fetal levels. ($n$) = number of samples per group.

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**Table 1** Effects of PNS and CORT on litter size, percent males, and maternal weight gain during treatment.

| Treatment group | Litter size (pups) | % Male (pups/litter) | Maternal weight gain E14–21 (g) |
|---|---|---|---|
| Oil | 9.7 ± 1.5 | 57.6 ± 8.8 | 71.1 ± 6.9 |
| PNS | 12.3 ± 1.1 | 54.0 ± 5.3 | 55.1 ± 5.2[a,b] |
| CORT | 11.6 ± 1.1 | 55.2 ± 4.8 | 86.3 ± 3.9 |

[a] $p < 0.05$ vs. oil.
[b] $p < 0.05$ vs. CORT.

E16 to E20, as the fetal adrenal glands began producing endogenous CORT (Dupouy et al., 1975). This is also reflected in the slight elevation of CORT in control dams from E16 to E20.

As there were no differences in CORT levels between unstressed saline- and oil-injected controls in the first experiment, only oil-injected animals were used as controls in subsequent experiments. Neither PNS nor CORT treatment altered litter size or the percentage of male pups per litter. PNS did, however, result in a significant decrease in maternal weight gain from E14 to E21, i.e., during the period of daily stress treatment, when compared to both the oil- and CORT-treated dams (Table 1).

### 3.2. GR mRNA and protein expression in the adult mPFC, hippocampus and hypothalamus

Adult expression of GR mRNA (Fig. 2A–C) and protein (Fig. 2D–F) were measured in the mPFC, hippocampus, and hypothalamus. One-way ANOVA for mRNA expression revealed significant treatment effects in all three brain regions (mPFC: $F_{(2,33)} = 5.76$, $p < 0.01$; hippocampus: $F_{(2,31)} = 8.74$, $p < 0.01$; hypothalamus: $F_{(2,35)} = 3.53$, $p < 0.05$). Post-hoc analyses showed that, in the mPFC and hypothalamus, GR mRNA was reduced in the CORT-treated group but not in the PNS group (Fig. 2A and C). In the hippocampus, both PNS and prenatal CORT exposure resulted in a significant reduction in GR mRNA expression compared to controls (Fig. 2B). GR protein levels were also significantly affected by the prenatal treatment (for mPFC: $F_{(2,21)} = 24.27$; for hippocampus: $F_{(2,30)} = 26.85$; for hypothalamus: $F_{(2,37)} = 15.61$; all $p < 0.01$). Further, post-hoc comparisons showed that GR protein expression was significantly reduced in all three brain regions by both PNS and CORT treatment compared to controls (Fig. 2D–F).

### 3.3. CRH expression in the adult hypothalamus

Both PNS and prenatal CORT treatment resulted in a significant reduction in CRH mRNA expression in the hypothalamus ($F_{(2,35)} = 7.36$; $p < 0.01$; Fig. 3A), accompanied by a 40–60% decrease in CRH protein ($F_{(2,39)} = 23.14$; $p < 0.01$; Fig. 3D).

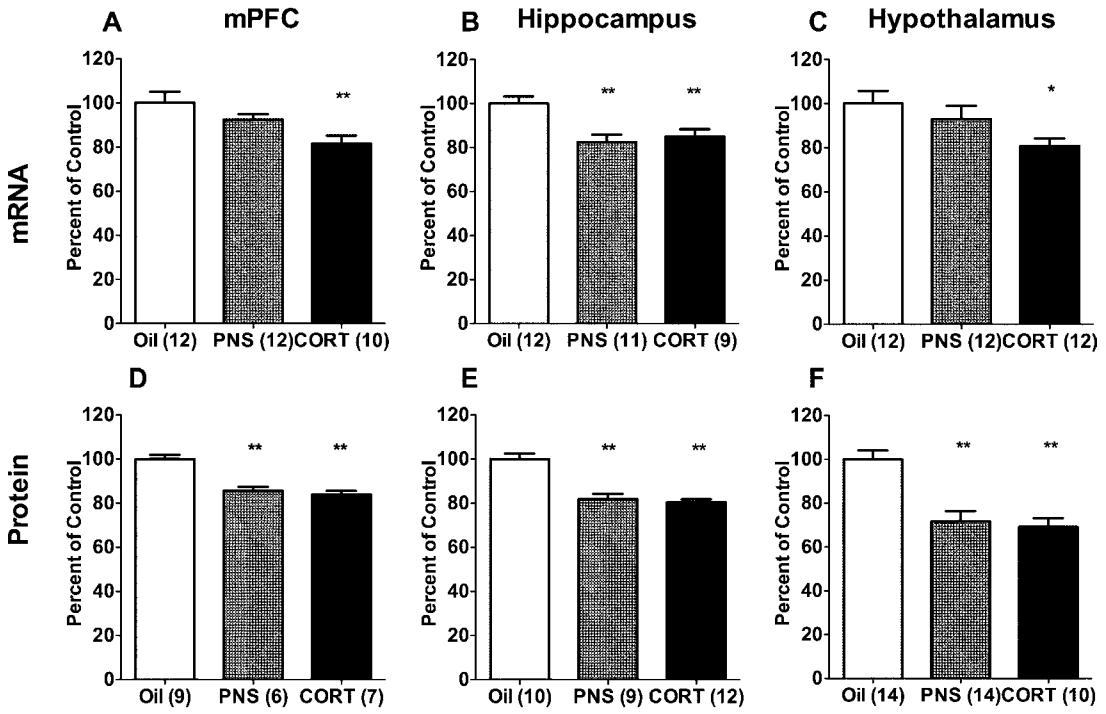


**Figure 2** Expression of GR mRNA and protein in the adult offspring of control, PNS, and CORT treated dams. Prenatal CORT treatment decreased GR mRNA expression in the mPFC (A), hippocampus (B) and hypothalamus (C). PNS decreased GR mRNA in the hippocampus only. Both PNS and CORT treatment decreased GR protein in all three regions (D–F). *$p < 0.05$, **$p < 0.01$ vs. control, ($n$) = number of samples per group.

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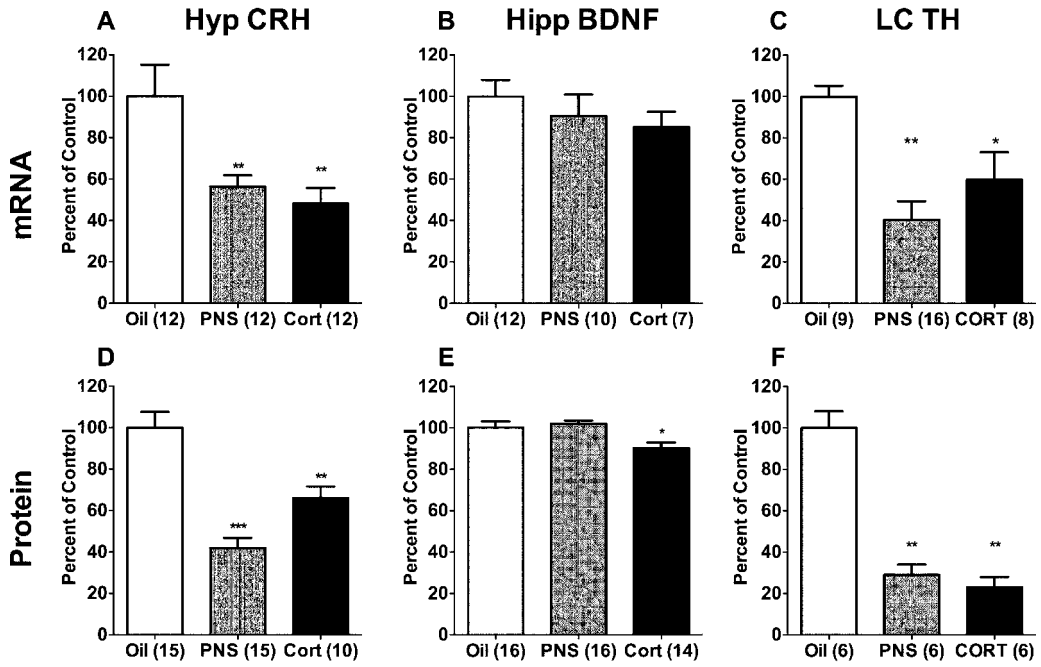


**Figure 3** Expression of CRH, BDNF, and TH in the adult offspring of control, PNS, or CORT treated dams. Both PNS and CORT treatment decreased CRH and TH mRNA and protein content in the hypothalamus (A and D) and LC (C and F), respectively. CORT treatment decreased hippocampal BDNF protein with no effect on mRNA (B and E). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. oil control, (*n*) = number of samples per group.

We also measured CRH mRNA expression in the adult hippocampus and found no change in either of the prenatal treatment groups compared to controls (data not shown).

### 3.4. BDNF expression in the adult hippocampus

No significant changes in BDNF mRNA expression were seen in the adult hippocampus in either treatment group ($F_{(2,28)} = 0.69$; $p = 0.5$; Fig. 3B). However, a modest but significant reduction in BDNF protein level was seen in the adult hippocampus of animals exposed to prenatal CORT treatment, but not to PNS ($F_{(2,45)} = 6.14$; $p < 0.01$; Fig. 3E).

### 3.5. TH expression in the adult locus coeruleus

ANOVA revealed a significant difference in the expression of TH mRNA ($F_{(2,33)} = 9.67$; $p < 0.01$; Fig. 3C) and protein ($F_{(2,17)} = 48.27$; $p < 0.01$; Fig. 3F) in the region of pons containing the LC. Post-hoc analyses indicate that both PNS and CORT treatment decreased TH expression when compared to control.

### 3.6. Fear conditioning and extinction

Having established that prenatal CORT treatment reproduces many of the effects of PNS on several neurobiological measures in adults, we next determined the impact of prenatal CORT treatment on fear conditioning and extinction behavior following adult CAPS stress (Fig. 4). CAPS stress alone increased freezing behavior during fear conditioning with no significant effect of prenatal CORT (Fig. 4A). A three-way ANOVA with repeated measures indicated main effects only for CAPS ($F_{(1,54)} = 5.99$; $p < 0.05$) and Tone ($F_{(3,162)} = 82.64$; $p < 0.01$).

By contrast, both CAPS and prenatal CORT significantly delayed the extinction of conditioned fear. A three-way ANOVA for freezing behavior during extinction learning (Fig. 4B) indicated main effects of prenatal CORT ($F_{(1,54)} = 5.22$; $p < 0.05$), CAPS ($F_{(1,54)} = 10.03$; $p < 0.01$), and Tone ($F_{(15,810)} = 37.13$; $p < 0.01$) with no significant interactions. The extinction learning impairment was also evident in the total amount of freezing displayed during the extinction procedure, measured by the area under the extinction curve (AUC). Two-way ANOVA for AUC again revealed main effects of prenatal treatment ($F_{(1,54)} = 4.77$; $p < 0.05$) and CAPS ($F_{(1,54)} = 10.65$; $p < 0.01$) with no interaction (Fig. 4C). Further, to better assess the rate and final degree of extinction, freezing behavior across tones was fit by non-linear regression analysis to a single exponential decay curve for each group (Fig. 4D). The decay constant (*k*) and plateau value were then analyzed. CAPS treatment significantly reduced the decay constant ($F_{(1,87)} = 5.25$; $p < 0.05$) while prenatal CORT tended to reduce the decay constant ($F_{(1,87)} = 2.67$; $p = 0.11$) (Fig. 4E). Neither factor significantly altered the plateau parameter (Fig. 4F).

Combined, these analyses indicate that CAPS and prenatal CORT independently impair acquisition of extinction learning by increasing total freezing during training and decreasing the rate of extinction learning without impacting the final level of asymptotic freezing behavior reached at the end of the extinction learning session.

One day after extinction learning, the animals were tested for their ability to recall the extinction training from the day before (Fig. 5). CAPS had no significant effect on extinction retention, whereas prenatal CORT treatment significantly impaired it (Fig. 5A). A three-way ANOVA indicated main effects of prenatal CORT ($F_{(1,54)} = 7.26$; $p < 0.01$), Tone ($F_{(15,810)} = 11.49$; $p < 0.01$), and a CORT by Tone interaction

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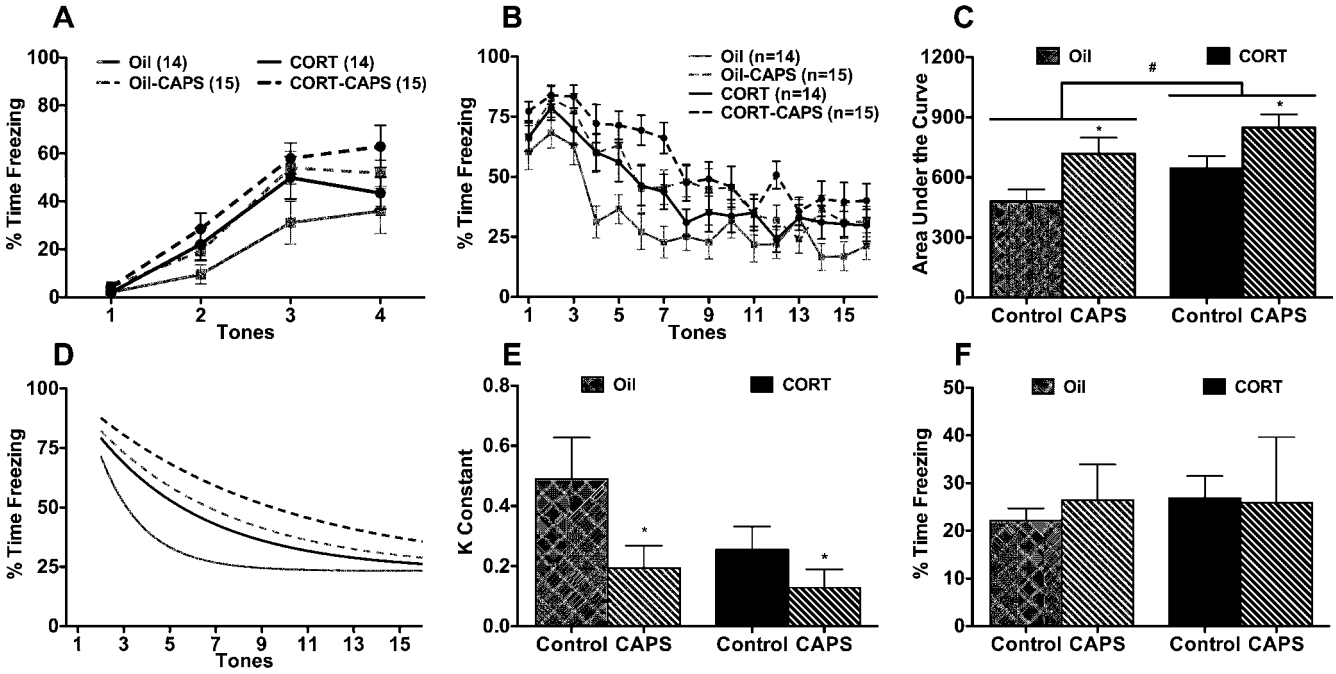


**Figure 4** Fear conditioning and extinction learning following prenatal CORT and adult CAPS treatment. (A) CAPS stress (dashed lines) significantly increased freezing behavior during fear conditioning. (B) Both prenatal CORT and adult CAPS treatment significantly delayed the extinction of cue-conditioned fear 3 days after conditioning. (C) Both PNS and CAPS increased total freezing behavior during extinction as measured by area under the curve. (D) Extinction data fitted to a single-exponential decay curve for each group. (E) Analysis of the decay constants ($k$) derived from the regression lines in (D) indicates that both prenatal CORT and adult CAPS stress reduced the rate of extinction learning. (F) By contrast, analysis of the plateau value indicates no effect of either prenatal CORT or CAPS on the final level of freezing behavior displayed at the end of the extinction learning session. $^{*}p < 0.05$ main effect of CAPS vs. control. $^{\#}p < 0.05$ main effect of prenatal CORT vs. control. ($n$) = number of subjects per group.

($F_{(15,810)} = 2.14$; $p < 0.01$). Animals treated prenatally with CORT also demonstrated significantly higher freezing in response to the first retention tone presentation (Fig. 5B), analyzed by 2-way ANOVA ($F_{(1,54)} = 6.45$; $p < 0.05$). Analysis of AUC as a measure of total freezing during extinction retention also showed a significant effect of prenatal CORT ($F_{(1,54)} = 7.17$; $p < 0.01$; Fig. 5C). When freezing behavior across tones was fit to a single exponential decay function (Fig. 5D), there were no significant effects of either CORT or CAPS on the rate constant; however, there was a main effect of prenatal CORT treatment on the plateau ($F_{(1,87)} = 15.27$; $p < 0.01$).

Combined, these analyses indicate that CORT-treated animals are able to re-extinguish at the same rate as controls; however, they are impaired in their initial extinction recall, and they are ultimately unable to extinguish to the same extent as controls (Fig. 5E and F).

## 4. Discussion

We previously demonstrated that PNS decreases GR and TH expression and impairs the extinction of learned fear (Green et al., 2011). The current study tested the hypothesis that fetal exposure to stress-relevant CORT levels is sufficient to recreate the programming effects of PNS on brain stress systems that also mediate fear learning and extinction. Pregnant dams were subjected to either PNS or daily injections of CORT, titrated to match the fetal CORT levels induced by PNS. The offspring were then allowed to grow to adulthood for neurochemical and behavioral testing. The results confirmed and extended our previous findings. Prenatal CORT exposure was sufficient to recapitulate PNS-induced decreases in GR expression in the mPFC, hippocampus, and hypothalamus of the adult offspring. Both prenatal treatments also decreased hypothalamic CRH and TH in the LC, whereas prenatal CORT alone decreased hippocampal BDNF. Prenatal CORT also mimics the previously described effect of PNS by impairing fear extinction and retention, alone and in combination with subsequent adult stress. With both prenatal stress and exogenous CORT treatment, circulating fetal CORT levels did not reach those measured in the dams, most likely due to placental metabolism of some proportion of maternal CORT before it could reach the fetus, by the enzyme, 11β-hydroxysteroid dehydrogenase type 2 (Seckl and Meaney, 2004). Maternal corticotrophin-binding globulin (CBG) may also have a role in regulating the amount of circulating CORT available to impact the fetus, and prenatal stress can decrease maternal CBG levels (Takahashi et al., 1998). However, this would, if anything, increase the amount of free CORT to which the fetuses could be exposed during stress, but is unlikely to have altered the relative levels of circulating CORT after exogenous administration. At any rate, similar fetal CORT levels were achieved in both conditions, and the results indicate that excessive CORT exposure during fetal development is sufficient for many of the neurochemical and behavioral consequences of PNS, including those related to associative fear extinction.

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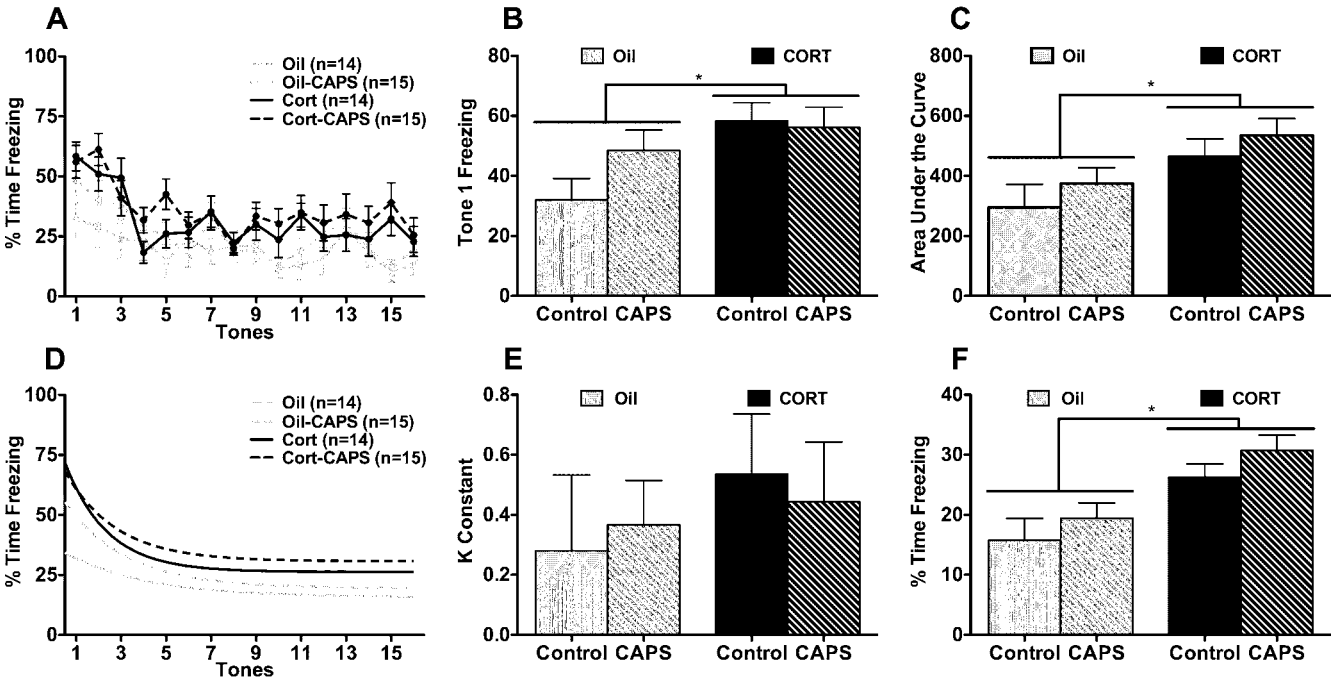


**Figure 5** Effects of prenatal CORT and adult CAPS treatment on the retention of extinction. (A) Prenatal CORT (black lines) significantly increased freezing behavior during extinction retention testing 24 h after extinction training. (B) Prenatal CORT significantly increased freezing behavior in response to the first retention tone presentation, indicating impaired recall of previous extinction training. (C) Only prenatal CORT increased total freezing behavior during extinction retention, as measured by area under the curve. (D) Extinction retention data fitted to a single-exponential decay curve for each group. (E) Analysis of the decay constants ($k$) derived from the regression lines in (D) indicates that all groups showed equivalent rates of extinction re-learning. (F) By contrast, analysis of the plateau term indicated that rats exposed to prenatal CORT treatment were unable to re-extinguish to the same final level of freezing behavior as controls. *$p < 0.05$ main effect of CORT vs. oil-treated group, ($n$) = number of subjects.

### 4.1. Long-term changes in gene and protein expression in the brain

Both PNS and CORT suppressed the expression of GR in multiple areas of the brain, providing evidence that fetal CORT exposure is the likely mechanism underlying the long-term programming effect of PNS on brain GR expression. PNS and prenatal CORT also decreased CRH mRNA and protein in the hypothalamus. As most hypothalamic CRH-containing neurons reside in the paraventricular nucleus (PVN), it is likely that the decrease occurred primarily in those HPA-related neurons. We previously found that PNS increased basal CORT secretion in the adult offspring (Green et al., 2011). Therefore, one potential interpretation of these data is that brain GR and CRH levels are down-regulated as a consequence of life-long exposure to elevated basal glucocorticoids. Alternatively, it is possible that the decreases in GR and CRH are established through direct epigenetic programming in utero and that the increase in basal CORT is a compensatory response. While changes in HPA regulatory proteins are not always noted with PNS or prenatal glucocorticoids, others have also found that PNS causes a decrease in negative feedback capability and an increase in basal or evoked glucocorticoid release (Barbazanges et al., 1996; Weinstock, 2008).

In human subjects, this pattern of CORT release and putative glucocorticoid sensitivity is more in line with a depressive-like phenotype than the traditional PTSD-like phenotype (Yehuda et al., 1991). However, two factors are important to consider. First, depression and PTSD are highly comorbid, and the associated HPA activity is often dependent on gender, trauma, and developmental stress history (Shea et al., 2005). Second, reductions in GR expression in the mPFC and hippocampus may have effects on stress-related learning and memory that transcend HPA regulation. GR agonist administration in the prelimbic subregion of the mPFC increases inhibitory avoidance memory and systemic administration of a GR antagonist blocks the reconsolidation of fear memory (Pitman et al., 2011; Roozendaal et al., 2009). GR activation increases the surface expression of both NMDA and AMPA receptors in the mPFC, facilitating short-term memory (Yuen et al., 2009). Activation of the mPFC, specifically the infralimbic (IL) subregion, also facilitates extinction learning and retention (Morgan et al., 1993; Vidal-Gonzalez et al., 2006). Therefore, it is possible that a decrease in GR expression within the mPFC and hippocampus may be more relevant to functional impairment of extinction learning and retention than to HPA regulation.

We also found that prenatal CORT caused a small but significant decrease in BDNF in the hippocampus. Hippocampal BDNF has been shown to play an important role in extinction learning (Peters et al., 2010). Thus the decrease in BDNF is consistent with the impairment in extinction retention in rats exposed to prenatal CORT. However, the reduction in BDNF was relatively modest, and there was no effect in PNS animals. Thus, while reduced BDNF expression may have contributed to the extinction deficit, it may not be

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the primary mechanism. Further, the differential effects on BDNF expression highlight the fact that prenatal CORT exposure is only one component of prenatal stress. It is unlikely that prenatal CORT can account for all of the long-term consequences of PNS.

Consistent with our previous study (Green et al., 2011), both PNS and prenatal CORT induced a long-term decrease in pontine TH mRNA and protein levels. This brain region contains the LC, the primary source of norepinephrine (NE) innervation of the forebrain, and the sole source of NE in the prefrontal cortex. Others have demonstrated a decrease in NE content, basal release, and nicotine-evoked release in the mPFC as a consequence of PNS (Carboni et al., 2010; Takahashi et al., 1992). However, the mechanisms underlying the long-term regulation of TH by prenatal CORT remain unclear. The TH gene contains a composite GRE/AP-1 site (Rani et al., 2009) and is responsive to regulation by glucocorticoids; however, this regulation is age- and brain region-specific. While direct comparison of fetal brain development in rats and humans varies by brain region, the period of maternal stress in this study (E14—E21) corresponds roughly to weeks 6—16 of human fetal brain development (Weinstock, 2001). A recent study demonstrated that glucocorticoids given to rats at days E18—E21 induced a marked *increase* in brainstem TH expression, TH activity, and brain NE content; however, when given postnatally, glucocorticoids had no effect (Kalinina et al., 2012). In adult animals, adrenalectomy blocked the stress-induced increase in TH expression in the nucleus of the solitary tract, whereas in the LC, exposure to CORT either had no effect or inhibited the stress-induced increase in TH expression (Makino et al., 2002; Núñez et al., 2009; Smith et al., 1991). Thus, the long-lasting reduction of TH expression may be due to epigenetic modification of transcriptional regulatory elements by prenatal CORT exposure, rather than direct transcriptional effects of glucocorticoids. Functionally, the long-term decrease in TH expression in the LC may reduce the capacity for NE release in the mPFC during extinction training.

### 4.2. Fear conditioning and extinction

Prenatal CORT and adult CAPS stress both altered fear conditioning and extinction in distinct patterns. CAPS stress increased freezing during fear conditioning, and delayed the acquisition of fear extinction, but had no further effect on the retention of extinction learning. By contrast, prenatal CORT exposure had no significant effect on freezing behavior during fear conditioning, but it also impaired extinction learning. Unlike CAPS stress, prenatal CORT also significantly impaired the recall of extinction learning and the final asymptotic level of freezing behavior that the animals achieved. The impairment of extinction by both prenatal CORT and adult CAPS stress, the lack of significant interaction between these factors, and differential effects on conditioning and extinction retention all suggest that the effects of prenatal CORT and adult stress are additive and independent. This is similar to our previous findings with PNS, in which CAPS and PNS both impaired extinction independently and additively with no interaction (Green et al., 2011). Hence, the combination of prenatal CORT exposure and adult stress creates an impaired phenotype that is greater than that created by either factor alone. These findings suggest that chronic or traumatic stress in humans, when superimposed on a history of high prenatal glucocorticoid exposure, can create an additive impairment on fear extinction — even in contexts that are distinct from the initial trauma. This type of extinction deficit is a prominent and defining feature of PTSD.

Effective extinction learning occurs, in part, through activity in both the IL subregion of the mPFC and the lateral amygdala. Within the lateral amygdala, extinction learning depends on NMDA receptor-mediated synaptic plasticity involving the glutamate NR2B receptor (Sotres-Bayon et al., 2007). In contrast, IL facilitation of extinction learning does not appear to be dependent on NMDA receptor activity (Santini et al., 2001). However, inactivation of the IL immediately prior to extinction training also results in impaired extinction learning and retention, indicating that it has an activity-dependent role in extinction learning (Sierra-Mercado et al., 2011). Therefore, it is possible that CAPS stress and/or prenatal CORT exposure impairs extinction learning either through a decrease in extinction-evoked IL activity or a decrease in NR2B-mediated plasticity in the lateral amygdala. In support of this hypothesis, a history of elevated CORT exposure has been shown to decrease both NMDA and AMPA receptors in the vmPFC and impair contextual extinction learning (Gourley et al., 2009).

In contrast to our model of chronic stress-induced impairments in extinction learning, others have found that acute single prolonged stress (SPS) impairs extinction learning and retention and is associated with an increase in GR expression within the prefrontal cortex and hippocampus (Knox et al., 2012). Although the authors did not distinguish between subregions of the PFC, similar behavioral outcomes might be achieved through differential modulation of GR expression patterns across PFC subregions that mediate the adaptive responses to specific types or durations of stress (Sotres-Bayon and Quirk, 2010).

The deficit in extinction retention that we previously described after PNS and currently describe in rats exposed to prenatal CORT may also be related to the reduced expression of TH in the LC and subsequent dysregulation of noradrenergic transmission. Specifically, such a deficit may be related to a reduced capacity for noradrenergic facilitation of extinction consolidation. NE is released during emotional arousal and helps mediate the consolidation of emotionally salient stimuli (Berlau and McGaugh, 2006). During extinction training, NE is released in the mPFC and promotes the consolidation of extinction memory through the activity of adrenergic $\beta_1$-receptors in the infralimbic (IL) region of the mPFC (Mueller et al., 2008). Release of NE during extinction facilitates the establishment of long-term potentiation such that upon subsequent extinction recall, the IL inhibits fear expression through projections to the basolateral (BLA) and intercalated (IC) nuclei of the amygdala (Amano et al., 2010; Knapska and Maren, 2009; Sierra-Mercado et al., 2011). Therefore, a putative decrease in extinction-induced release of NE in the IL, as a result of prenatal CORT exposure, could impair consolidation of the extinction memory. Norepinephrine has also been shown to interact with glucocorticoids in the mPFC to establish emotionally salient memories (Barsegyan et al., 2010). Therefore, the deficits in extinction learning and retention, noted especially in the prenatal CORT—CAPS animals, may be due to a decrease in mPFC GR acting in concert with a putative decrease in NE release

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capability. Because we previously demonstrated that CAPS stress alone has no effect on TH expression, this may also explain why CAPS stress alone is insufficient to impair extinction retention. As such, these animals may be delayed in acquiring extinction, but once acquired, they have sufficient NE release to consolidate that learning. Deficits in extinction learning and retention after other types of adult stress have also been associated with an IL-specific retraction of apical dendrites and a switch from IL-driven long-term depression to long-term potentiation within the BLA (Izquierdo et al., 2006; Maroun, 2006), either of which could potentially alter IL-mediated inhibition of the BLA and impair extinction of learned fear.

It is possible that PNS could have altered post-natal maternal behavior toward the pups, to account for some of the long-term behavioral changes in the adult offspring, although this is less likely for prenatal CORT treatment alone. Others have shown that PNS decreased nursing time and increased the time the dam spent away from the nest, but had no effect on more consequential licking and grooming behaviors (Bourke et al., 2013). Cross-fostering PNS pups to non-stressed mothers reversed some of the long-term behavioral and neurobiological effects of PNS, although these findings are complicated by effects of early cross-fostering itself, independent of PNS (Maccari et al., 1995). Prenatal CORT administration has been shown to alter maternal behavior, but only at doses much higher (40 mg/kg) than those used in the current study, whereas CORT at the dose we gave (10 mg/kg) had no effect (Brummelte and Galea, 2010).

## 5. Conclusion

We have demonstrated that prenatal CORT exposure, at levels comparable to those achieved during prenatal stress, significantly altered the long-term expression of several proteins crucial to the adult stress response in stress-relevant brain regions, replicating and expanding upon previously demonstrated effects of prenatal stress. Further, prenatal CORT exposure also reproduced the previously reported effects of PNS on the extinction of cue-conditioned fear behavior. These results suggest potential mechanisms that link the molecular programming effects of prenatal CORT exposure within components of the brain stress response system to changes in fear-related learning and plasticity. The manner in which two independent risk factors (e.g., prenatal CORT exposure and adult stress) combine to dysregulate fear responding may inform our understanding of the mechanisms underlying susceptibility and development of stress-related psychiatric disorders such as PTSD. Further, the resulting inability to extinguish learned fear responses may exacerbate exposure to secondary stressors associated with the trauma as susceptible individuals continue to respond to fear-inducing cues. This may facilitate the transition from acute stress disorder to chronic PTSD, and limit the subsequent efficacy of extinction-based therapies. This research further highlights the need for effective interventions following traumatic stress, particularly for individuals who may be at risk due to early life history.

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## Conflict of interest

Dr. Frazer has served on advisory boards for Lundbeck and for Takeda Pharmaceuticals International, Inc. and Eli Lilly and Co. Dr. Morilak has received research funding from Forest Labs and Lundbeck. None of these activities represents a conflict with the present work. All other authors declare that they have no conflict of interest.

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